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ᴌUPERT E. BILLINGHAM, a Member of The Wistar
titute and Professor of Zoology at the University of
ınsylvania, holds a D.Sc. from Oxford and is a Fellow
the Royal Society. Before taking up his present ap-
ıtment in 1957, Dr. Billingham worked at University
ege London, England where he collaborated with
fessor Peter B. Medawar, Dr. Leslie Brent and others
studies on mammalian pigmentation, the preservation
tissues for transplantation, wound healing, and the
nomena of tissue transplantation immunity, including
ft-versus-host reactions, and immunological tolerance.

VILLYS K. SILVERS is a mammalian geneticist who
eived his doctoral training in the Department of
logy at the University of Chicago, after which he was
the staff at the Roscoe B. Jackson Memorial Labora-
y at Bar Harbor, Maine. Dr. Silvers is currently an
ociate Member of The Wistar Institute and a Research
ociate in the Department of Dermatology at the Uni-
sity of Pennsylvania. He has employed transplanta-
ı techniques in important analyses of the mode of action
nammalian coat color determinants at the cellular level.
ring the past 4 years, genetic aspects of tissue homo-
ft compatibility and immunological tolerance in various
cies have constituted his major fields of activity.

ᴾAUL S. RUSSELL is Associate Professor of Surgery
the College of Physicians and Surgeons of Columbia
iversity, New York. He obtained his M.D. at the Uni-
sity of Chicago and received his training in surgery at
ssachusetts General Hosptal. Before moving to Colum-
University, Dr. Russell was a Clinical Associate at
rvard Medical School. His published papers include
dies on tissue homotransplantation, with special refer-
es to endocrine tissues, and on wound healing.

ᴊOY PALM received her doctoral training in immuno-
etics in the Genetics Department of the University of
sconsin where she studied cellular antigens of avian
cies hybrids and of cattle twins. Before joining the
f of The Wistar Institute, Dr. Palm held appoint-
ıts at the Roscoe B. Jackson Memorial Laboratory,
r Harbor, Maine, and the Sloan-Kettering Institute,
v York. Her present research is concerned with im-
nogenetic aspects of tissue transplantation, including
relationship between hemagglutinogens and histo-
ıpatibility antigens.

). BERNARD AMOS received his training in medicine
Guy's Hospital, London, England. He subsequently
l a Senior Research Fellowship in the Department of
hology, where he worked partly in collaboration with
late Dr. Peter A. Gorer. His research contributions
e been in the fields of tumor immunology, mouse gen-
s and the differentiation of antigens. Dr. Amos is
rently a Principal Cancer Research Scientist in the
artment of Experimental Biology, Roswell Park Me-
ial Institute, Buffalo, New York.

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e Effect of n-Butyl Alcohol on the Permeability of ythrocytes to Non-Electrolytes

F. R. HUNTER[1]
Department of Biology, University of the Andes,
Bogotá, Colombia

The effect of n-butyl alcohol on erythro- es has been studied by a number of vestigators. For example, Netsky and obs ('39) suggested that hemolysis in presence of this alcohol resulted from cells becoming cation permeable. Pon- ('48) showed that hypolytic concen- tions of butanol increased the perme- lity of erythrocytes to potassium and rpart and Green ('51) demonstrated an ual exchange of Na for K preceding molysis in butanol-treated red cells. Ja- bs ('51) suggested that butanol not only creases the permeability of the erythro- e to cations but also decreases their ion permeability. It has been suggested arpart and Ballentine, '51; Parpart and een, '51) that butanol in some way ers the lipo-protein complex in the cell mbrane.

Since such a change might be expected alter the aqueous channels in the mem- ane, the present study was undertaken determine the effect of butanol on the rmeability of the erythrocyte to non- ctrolytes.

GENERAL PROCEDURES

Chicken and rabbit blood were obtained cardiac puncture; human and sheep ood by venipuncture. Heparin or citrate s used as anticoagulant. The cells were shed two or three times with Ringer- cke solution (RL) or with a NaCl-phos- ate solution (A) (9 parts 0.9% NaCl s one part of a mixture of 0.11 M H_2PO_4 and 0.11 M Na_2HPO_4; pH 7.0). lls stored in the refrigerator were shed an additional time immediately fore being used. Measurements of rate hemolysis or of volume changes were ade with the same apparatus previously scribed (Hunter, '60). To measure the rate of entrance of a substance: (1) The rate of swelling was measured by adding erythrocytes to 8 cm^3 of a 0.3 M solution of non-electrolyte in RL (or in A) or 8 cm^3 of M/6 NH_4Cl in RL (or in A); or (2) cells were added to 8 cm^3 of 0.3 M non-electrolyte in water or M/6 NH_4Cl in water. To measure the rate of exit of a substance, cells were equilibrated in a 0.3 M solution of non-electrolyte in RL (or in A) or in M/6 NH_4Cl in RL (or in A). These suspensions were then centrifuged (5 minutes at 1200 × g) and the supernatant fluid removed. A measured volume of these cells was then added to 8 cm^3 of 1.75 × RL (or 1.75 × A) in the densimeter. All measurements were made at room temperature (22–25°C). Na and K measurements were made using the internal standard method with a Perkin-Elmer Flame Photometer (cf. Hunter et al., '56). A Beckman pH meter was used for adjusting the pH of the solutions.

Initial chronic experiments.[2] Control suspensions were prepared by mixing equal volumes of washed rabbit erythrocytes and RL. Experimental suspensions were similarly prepared using 0.25 or 0.35 M n-butyl alcohol in RL. Both the rate of entrance (swelling) and the rate of exit (shrinking) were measured. In the majority of the experiments there were no differences between control and experimental cells. In a few experiments, however, differences were observed. For example, in one series of observations the average times in seconds for one half the total deflections were as follows: swelling in glycerol—control, 18.7; experimental, 20.6; swelling in thiourea—control, 54.2; experimental, 40.0. These data suggested the rather unlikely possibility that butanol was decreasing the rate of entrance of glycerol but increasing the rate of entrance of thiourea.

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[1] Acknowledgment is made to the Rockefeller Foundation and to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for the support of this research.

[2] The author wishes to acknowledge the help of W. Bruce Hunter with these experiments.

Salt analyses. The lack of effect of butanol in the majority of the experiments in this initial chronic series might have resulted from one or two factors: (1) Under the conditions of the present experiment butanol was not altering the membrane, or (2) An alteration was rapidly reversed. To test the first point, Na and K measurements were made on the stock suspensions. In one experiment, for example, after two hours the K was 61 meq/l of cells and after 24 hours, 48 meq/l. In other experiments, however, there was little exchange of Na for K during the time the permeability measurements were made.

This being the case, the method was changed to that used by Parpart and Green ('51) in which an isotonic NaCl solution buffered with isotonic phosphate (solution A) was used in place of Ringer-Locke solution. Table 1 shows typical data obtained using this solution.

The first experiment illustrates that under appropriate conditions it was possible to obtain rabbit red cells in which an almost complete exchange of Na for K had occurred as was reported by Parpart and Green. In the second experiment, in which two different volumes of the same cell suspension were used, it can be seen that when the ratio between volume of cell suspension and volume of buta solution is smaller, the cations exchan more rapidly. This explains why 0.4 butanol is hemolytic in a few minu when the relative proportion of cells very small as, for example, in expe ments to be reported subsequently.

On the basis of these salt analyses can be said that in the experiments to described, with the higher concentratio of butanol, a rapid exchange of Na for occurs. In the experiments with low concentrations of butanol, the exchan is probably negligible during the sh time that is necessary to make the perm ability measurements.

Data to be presented subsequently sh that the effect of butanol on non-elect lyte permeability is rapidly reversed si ply by resuspending the cells in a soluti free of butanol. This, then explains t difficulty in demonstrating an effec butanol with a chronic experiment. soon as the small volume of the stock su pension is added to the relatively lar volume of solution in the chamber of t densimeter, the butanol is greatly dilut and its effect is almost completely revers immediately.

Acute experiments: initial survey. this series of experiments the rate penetration of glycerol, monoacetin, t ourea and NH_4Cl was measured in a 0.3 non-electrolyte solution in RL or in M NH_4Cl in RL and in the same so tions containing 0.35 M n-butyl al hol. Typical results obtained in a sin experiment using rabbit blood are sho in figure 1. It can be seen that in t presence of butanol, the rate of swelling

TABLE 1

Na and K content of rabbit erythrocytes after exposure to n-butyl alcohol

Suspension	Time	meq/l (corrected to initial volume of cells)			
		K		Na	
		Control	Exp.	Control	Exp.
	min.				
1 cm³ cells plus 20 cm³ 0.4 M butanol	30	107	4	20	106
1 cm³ cells plus 20 cm³ 0.4 M butanol	30	116	55	32	63
2 cm³ cells plus 20 cm³ 0.4 M butanol	30	122	100	15	34

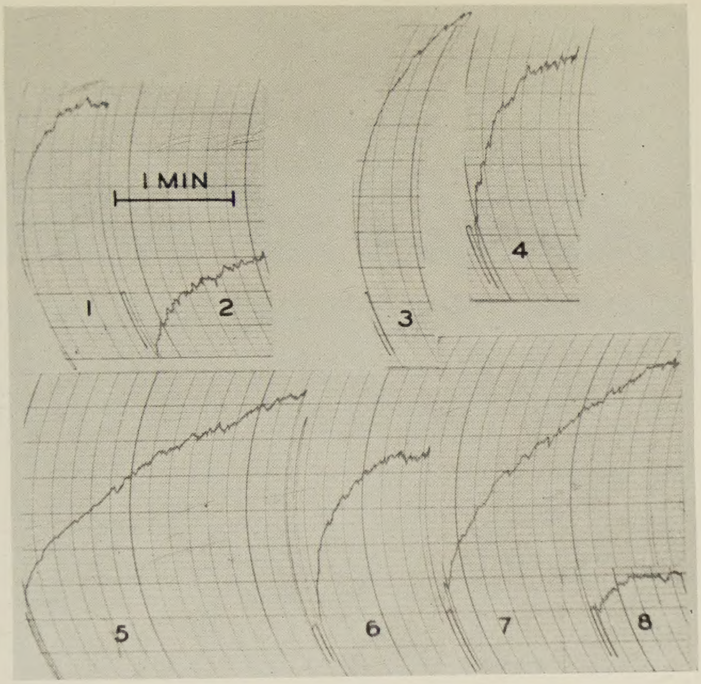


Fig. 1 The effect of 0.35 M n-butyl alcohol on the rate of swelling of rabbit erythrocytes. 1, 0.3 M glycerol in RL; 2, 0.3 M glycerol in RL plus 0.35 M butanol; 3, 0.3 M monoacetin in RL; 4, 0.3 M monoacetin in RL plus 0.35 M butanol; 5, 0.3 M thiourea in RL; 6, 0.3 M thiourea in RL plus 0.35 M butanol; 7, M/6 NH_4Cl in RL; 8, M/6 NH_4Cl in RL plus 0.35 M butanol.

cerol, monoacetin and NH_4Cl is slower in thiourea it is faster. Considering y the glycerol and thiourea data, a sible basis for an explanation of the osite action of butanol might be found the fact that thiourea enters rabbit throcytes by diffusion whereas an ac- e process is involved in the penetration glycerol (see, for example, Le Fevre,). To investigate this possibility, a re intensive study was made of the netration of glycerol.

Glycerol. The effect of different con- ntrations of butanol and the reversibility any observed change was first studied ng rabbit blood. It can be seen from ure 2 that the rate of swelling in 0.3 M cerol in A is decreased with increasing ncentrations of butanol (curves 1–6). ter the swelling was completed, the cell spensions were removed from the cham- r of the densimeter, centrifuged (5 nutes, 1000 × g) and the supernatant id removed. These same cells, without further washing, were then added to 8 cm^3 of 1.75 × A in the chamber of the densimeter, and the rate of shrinking measured (curves 7–12). With the exception of the cells in 0.4 M butanol, the rate at which glycerol left the cells was the same with control and experimental cells indicating the complete and rapid reversal of the butanol effect. In some experiments, the cells in 0.4 M butanol were so hemolyzed that shrinking measurements could not be made (cf. paragraph on *Salt analyses*).

To make a similar study of the effect of increasing concentrations of butanol on the rate at which glycerol leaves the cells, washed rabbit erythrocytes were suspended in 0.3 M glycerol in A. After 15–30 minutes, this suspension was centrifuged (5 minutes, 1000 × g) and the supernatant liquid removed. An aliquot of these cells was added to 8 cm^3 of 1.75 × A plus butanol (0–0.4 M) in the chamber of the densimeter. Following the shrinking measurements, the suspensions were removed and centrifuged and the cells added to 8 cm^3 of 0.3 M glycerol in A for measurements of swelling. It can be seen from figure 3 that with the exception of 0.4 M, increasing concentrations of butanol decrease the rate at which glycerol leaves the cells. This change is rapidly and completely reversible, except in the case of 0.4 M and possibly 0.2 M butanol.

As a final test of the effect of conce
tration of butanol, hemolysis measu
ments were made. Rabbit erythrocyt
(0.02 cm^3) were added to 8 cm^3 of 0.3
glycerol plus butanol (0–0.4 M) in t
chamber of the densimeter. Once again
can be seen (fig. 4) that with the exce
tion of 0.4 M, increasing concentratio
of butanol increase the time for hemoly
in glycerol.

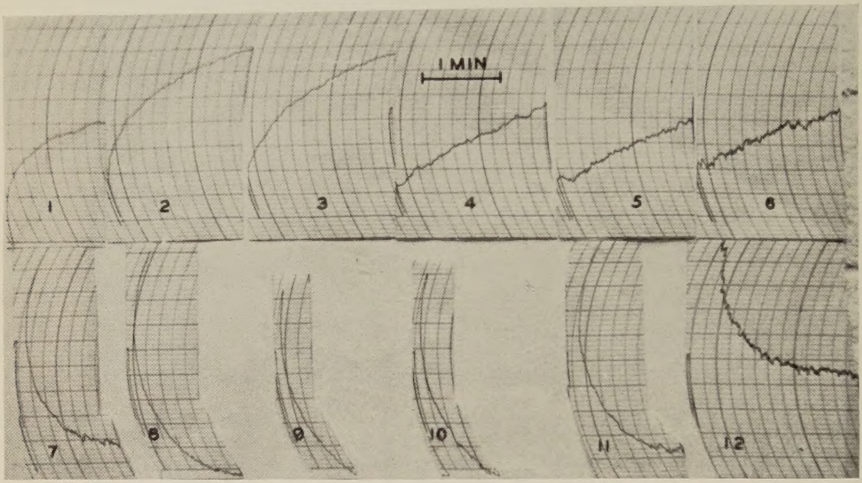


Fig. 2 The effect of concentration of butanol on the rate of swelling of rabbit erythrocytes in 0.3 M glycerol in A and the reversibility of these changes. 1,7, Control; 2,8, 0.02 M; 3,9, 0.04 M; 4,10, 0.1 M; 5,11, 0.2 M; 6,12, 0.4 M.

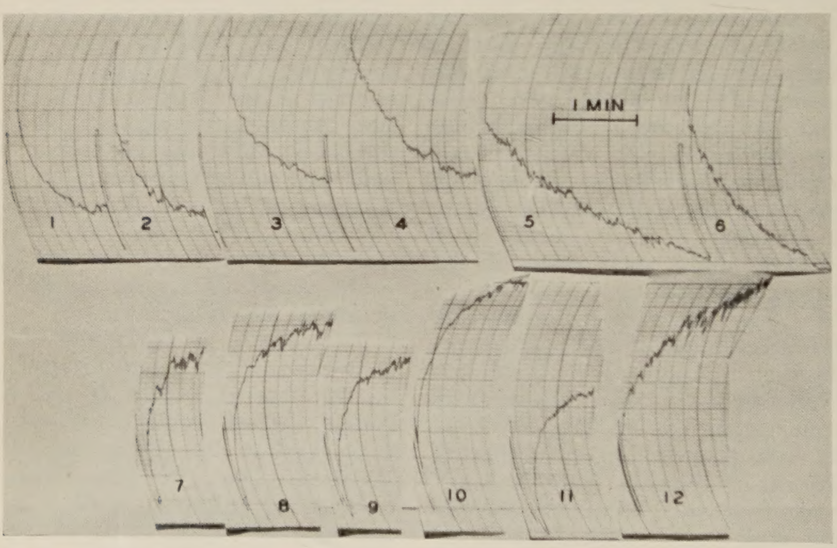


Fig. 3 The effect of concentration of butanol on the rate of shrinking of rabbit erythrocytes in 1.75 × A (previously equilibrated in 0.3 M glycerol in A) and the reversibility of these changes. 1,7, Control; 2,8, 0.02 M; 3,9, 0.04 M; 4,10, 0.1 M; 5,11, 0.2 M; 6,12, 0.4 M.

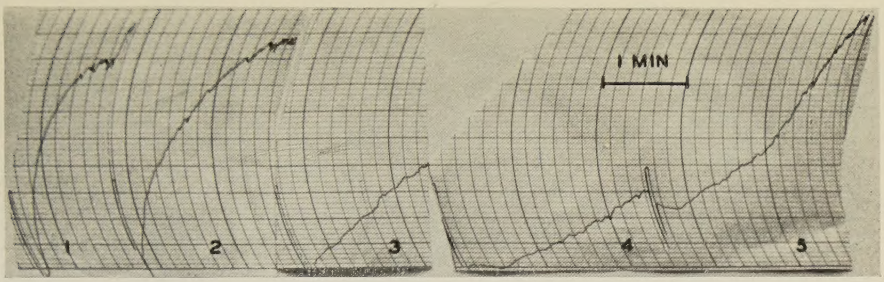


Fig. 4 The effect of concentration of butanol on the rate of hemolysis of rabbit erythrocytes in 0.3 M glycerol in water. 1, Control; 2, 0.02 M; 3, 0.1 M; 4, 0.2 M; 5, 0.4 M.

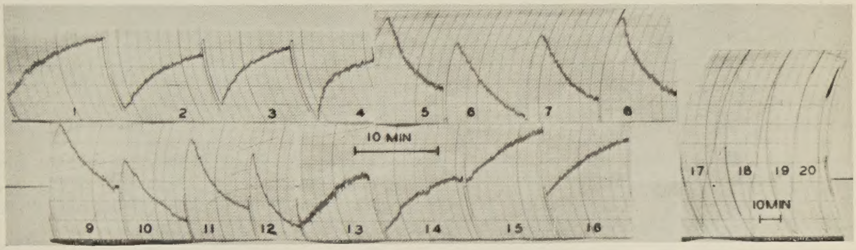


Fig. 5 The effect of butanol on the permeability of chicken erythrocytes to glycerol and the reversibility of these changes. 1–4, Swelling in 0.3 M glycerol in A (1, control; 2, 0.02 M; 3, 0.1 M; 4, 0.2 M). 5–8, Reversibility; shrinking in 1.75 × A (same sequence as in 1–4). 9–12, Shrinking in 1.75 × A following previous equilibration in 0.3 M glycerol in A (same sequence as in 1–4). 13–16, Reversibility; swelling in 0.3 M glycerol in A (same sequence). 17–20, Hemolysis in 0.3 M glycerol in water (same sequence).

Having shown that with rabbit erythro- tes, increasing concentrations of butanol ogressively decreased their permeability glycerol in a reversible manner, a simi- r series of experiments was performed ing chicken erythrocytes in which there no evidence of an active component in ycerol permeability. Data for swelling, rinking and hemolysis are presented in ure 5. With each group of 4 curves the der is always the same—control, 0.02 M tanol, 0.1 M butanol and 0.2 M butanol. rves 1–4 show the increase in rate of elling with an increase in the concentration of butanol. Curves 5–8 show the versibility of this effect. (Same cells used 1–4 added to 1.75 × A following cen- ifugation.) The acceleration of the rate exit of glycerol with increasing concen- ations of butanol can be seen in curves –12. That this effect also is reversible n be seen from curves 13–16. (Cells of –12 placed in 0.3 M glycerol in A follow- g centrifugation.) Finally, curves 17– show the increase in rate of hemolysis ith increasing concentrations of butanol.

The fact that butanol reversibly increases the permeability of chicken erythrocytes to glycerol but reversibly decreases the permeability of rabbit red cells, lent more support to the hypothesis that in rabbit red cells, the butanol was in some way interfering with the active movement of glycerol across the membrane. The observation of Jacobs et al. ('35) that acid pH's decrease the rate of hemolysis in glycerol of rabbit erythrocytes suggests the possibility that at acid pH's the active movement of glycerol is decreased. It was of interest, then, to see what effect butanol would have on the rate of penetration of glycerol at different pH's. Solutions of 0.3 M glycerol in A were adjusted to various pH's by the addition of a drop or two of concentrated NaOH or HCl. Such small quantities of acid or base were required, the osmotic effect of these additional ions was negligible. Similar solutions containing 0.1 M butanol were also prepared. It was found that a pH as acid as 3.4 rapidly

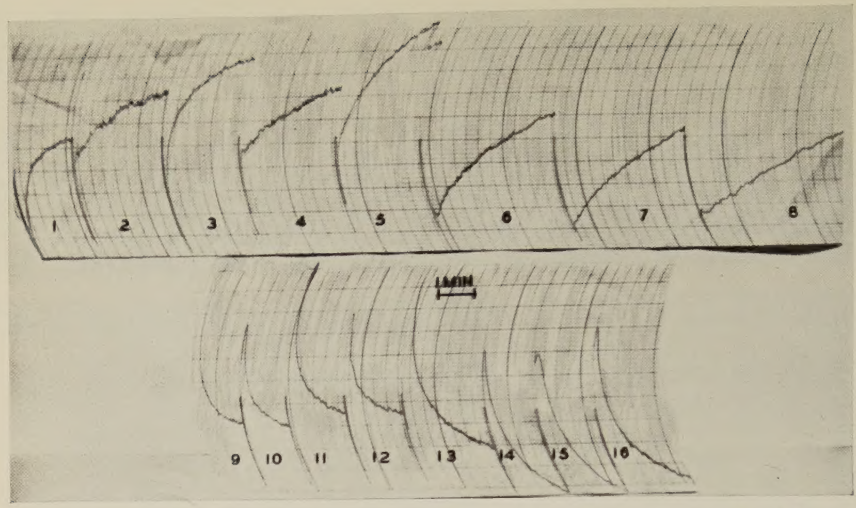
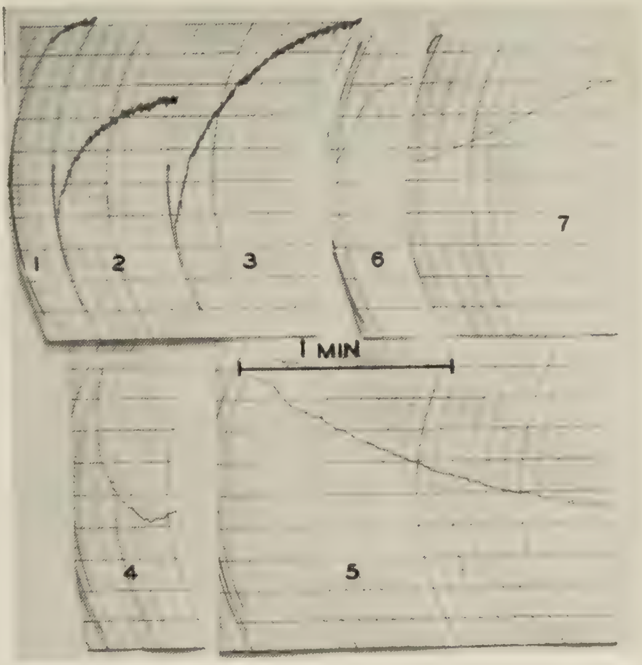


Fig. 6 The effect of pH and n-butyl alcohol on the rate of swelling of rabbit eryt cytes in 0.3 M glycerol in A. 1,9, pH 8.0; 2,10, pH 8.0 plus 0.1 M butanol; 3,11, pH 7. 4,12, pH 7.0 plus 0.1 M butanol; 5,13, pH 5.5; 6,14, pH 5.5 plus 0.1 M butanol; 7,15, p 4.9; 8,16, pH 4.9 plus 0.1 M butanol.

damaged the cells. The results of a typical experiment can be seen in figure 6. The rates of swelling at pH 8 and 7 are essentially the same. At pH 5.5 there is some inhibition and at pH 4.9 there is considerable inhibition. At the 4 pH's, the rate of swelling in the presence of butanol was always slower than at the same pH without butanol. But the rate of swelling in the presence of butanol was not affected by pH to any great extent. Or to put it differently, at pH 4.9 butanol had much less relative effect than at pH 7 or 8. In several experiments, as in the one shown, the rate of swelling at pH 5.5 in the presence of butanol was more rapid than at pH 7 in the presence of butanol. The reversibility of these effects is shown by curves 9–16. (Cells from curves 1–8, centrifuged and added to 1.75 × A.) The effect of pH 4.9 was not completely reversible. The significance of the more complete reversal at pH 4.9 in the presence of butanol is unknown but this was observed in all of these experiments.

Although these experiments at different pH's did not conclusively demonstrate that the butanol was interfering with the active movement of glycerol, the results obtained could certainly be explained using this hypothesis. Since copper is known to decrease markedly the rate of hemolysis in glycerol of human erythrocytes (Jacobs and Corson, '34), and since this effect ca be reversed using sulfhydryl agents as cysteine (e.g., Le Fevre, '48), another series of experiments was undertaken u ing this system. Before doing this, how ever, the effect of various concentration of butanol and different pH's, us man red cells, was studied. The were qualitatively similar to the tained with rabbit blood, but q tively, the changes were greater. I 7 are a few typical results which strate this quantitative difference. Cur 1 shows the rate of swelling of contr cells in glycerol at pH 7, curve 2, the i hibition due to 0.1 M butanol at pH 7 ar curve 3, the slower rate of penetratio of glycerol at pH 5.5. As with rabb blood, acid pH's (5.5 and 4.9) inhibit the rate of swelling in glycerol. On tenth molar butanol at all pH's i hibited but the inhibition was relative less at acid pH's. With human blood, t effect of both pH 5.5 and 4.9 was n completely reversible but the effect 0.1 M butanol at these pH's was mo nearly completely reversible than the p effect alone. Curves 4 and 5 show t effect of 0.1 M butanol on the rate rinking of cells previously equilibrated glycerol. With the exception of 0.4 M tanol, there was a direct relationship etween concentration of butanol and de-ree of slowing of the rate of shrinking .2 > 0.4 > 0.1 > 0.04 > 0.02). Also, with the exception of 0.4 M, the effect was completely reversible. Curves 6 and 7 show the effect on hemolysis of 0.1 M butanol. Once again, there was a direct correlation with concentration of butanol (0.2 > 0.1 > 0.02).



ig. 7 A few typical results showing the effect of pH and butanol on the permeability human erythrocytes to glycerol. 1, Swelling in 0.3 M glycerol in A, pH 7.0; 2, same plus M butanol; 3, swelling in 0.3 M glycerol in A, pH 5.5; 4,5, shrinking in 1.75 × A after vious equilibration in 0.3 M glycerol in A (4, control; 5, 0.1 M butanol). 6,7, Hemolysis .3 M glycerol in water (6, control; 7, 0.1 M butanol).

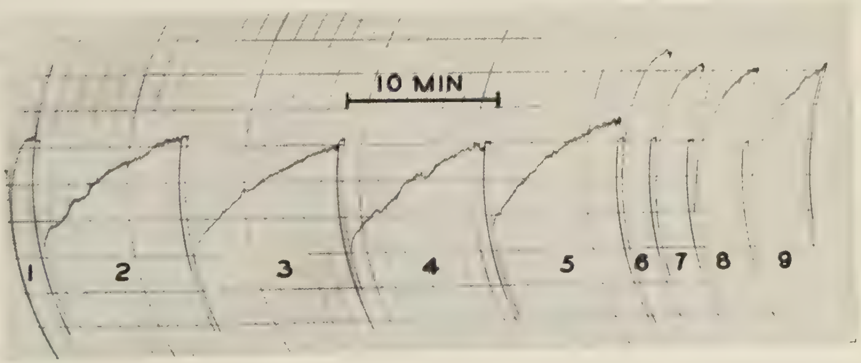


Fig. 8 The rate of swelling of human erythrocytes in 0.3 M glycerol in A as affected by the presence of copper, copper plus cysteine and butanol. 1, Normal control; 2, 10^{-4} M $CuCl_2$; 3, 10^{-4} M $CuCl_2$ plus 0.01 M butanol; 4, 10^{-4} M $CuCl_2$ plus 0.05 M butanol; 5, 10^{-4} M $CuCl_2$ plus 0.1 M butanol; 6, 10^{-4} M $CuCl_2$ plus 6×10^{-4} M cysteine; 7, 10^{-4} M $CuCl_2$ plus 6×10^{-4} M cysteine plus 0.01 M butanol; 8, 10^{-4} M $CuCl_2$ plus 6×10^{-4} M cysteine plus 0.05 M butanol; 9, 10^{-4} M $CuCl_2$ plus 6×10^{-4} M cysteine plus 0.1 M butanol.

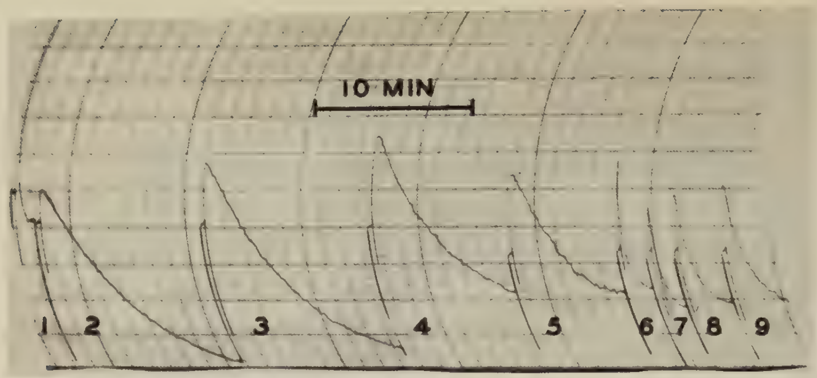


Fig. 9 The rate of shrinking of human erythrocytes (previously equilibrated in 0.3 M glycerol in A) in 1.75 × A as affected by the presence of copper, copper plus cysteine and butanol. As in figure 8, the final concentration of $CuCl_2$ was 10^{-4} and of cysteine, 6×10^{-4} M. 1, Normal control; 2, copper; 3, copper plus 0.01 M butanol; 4, copper plus 0.05 M butanol; 5, copper plus 0.1 M butanol; 6, copper plus cysteine; 7, copper plus cysteine plus 0.01 M butanol; 8, copper plus cysteine plus 0.05 M butanol; 9, copper plus cysteine plus 0.1 M butanol.

Having determined that butanol affected human cells in a manner similar to its effect on rabbit cells, the experiments with copper were undertaken. In figure 8, the results of a typical experiment can be seen. Curve 1 shows the rate of swelling in 0.3 M glycerol in A. Curve 2 shows the inhibition resulting from a final concentration of $CuCl_2$ of 10^{-4} M. Curves 3–5 show the effect of the addition of 0.01 M, 0.05 M and 0.1 M butanol respectively in the presence of 10^{-4} M $CuCl_2$. A careful study of these curves shows an acceleration in the rate of swelling, increasing with increasing concentrations of butanol. To obtain the last 4 curves, the glycerol solution contained 10^{-4} M $CuCl_2$ plus 6×10^{-4} M cysteine. As can be seen from curve 6, the cysteine completely reversed the Cu-inhibition. Curves 7–9 demonstrate the increasing inhibition with increasing concentrations of butanol (0.01, 0.05 and 0.1 M). This experiment demonstrates that when the active movement of glycerol is inhibited by copper, butanol accelerates the rate of penetration of glycerol. When, however, the effect of copper is reversed by cysteine, butanol inhibits the active penetration of glycerol.

The same experiment was then performed to study the rate at which glycerol left the cells. Cells were first equilibrated in 0.3 M glycerol in A and then centrifuged. These cells were then added to 1.75 × A in the densimeter. The firs curve in figure 9 shows the normal rapi rate of shrinking as glycerol leaves th cells. In the second curve, the inhibitin effect of copper can be seen. Curves 3– have the same concentration of coppe plus 0.01, 0.05 and 0.1 M butanol respectively. As with the swelling experiment it can be seen that increasing concentrations of butanol increase the rate of diffusion of glycerol from the cells. In th last 4 experiments, the effect of coppe has been reversed by the addition o cysteine. Increasing concentrations of butanol (curve 6, none; 7, 0.01 M; 8, 0.0 M; 9, 0.1 M) decrease the rate at whic glycerol leaves the cells.

Finally, hemolysis experiments wer performed (fig. 10). In this case the accelerating effect of butanol in the presenc of copper is most easily seen (curves 3–5) Cysteine only partially reversed the coppe effect (curve 6).

Considering all of the evidence presented, one can say that increasing concentrations of butanol increase the rate a which non-electrolytes diffuse into erythrocytes. In the case of active movemen of glycerol, increasing concentrations o butanol decrease the rate of transport.

If this is really the case, then one shoul be able to make observations using rabbi erythrocytes similar to those reported wit human red cells. Since the copper effec less obvious with rabbit erythrocytes acobs et al., '35, p. 212), it might be pected that the experiments using rabbit lls would be less clear cut, as was the se. Since higher concentrations of cop- er were necessary to inhibit the active ovement of glycerol into rabbit red cells, n immediate problem was encountered ith shrinking and swelling experiments ecause of the formation of a precipitate $Cu_3(PO_4)_2$. However, clear cut results ere obtained with hemolysis experiments s illustrated in figure 11. Curves 4–6 early demonstrate the accelerating ef- cts of increasing concentrations of buta- ol in the presence of copper. Curve 7 lustrates the partial reversal obtained ith cysteine.

To determine whether or not butanol nd copper exerted their effect through a milar mechanism, attempts were made to see if cysteine would reverse the butanol inhibition of glycerol transport. Typical data obtained with both human and rabbit erythrocytes are presented in figure 12. Since the rate of movement of glycerol in the presence of different concentrations of butanol is the same whether or not cysteine is present, it can be concluded that butanol acts in a different way than copper.

As a final control, a similar series of experiments was performed using sheep erythrocytes in which there is no evidence of active movement of glycerol. As can be seen in figure 13, increasing concentrations of butanol accelerate the rate of entrance of glycerol and the rate at which it leaves these cells. Copper does not inhibit.

To summarize, it can be seen that when the movement across the cell membrane

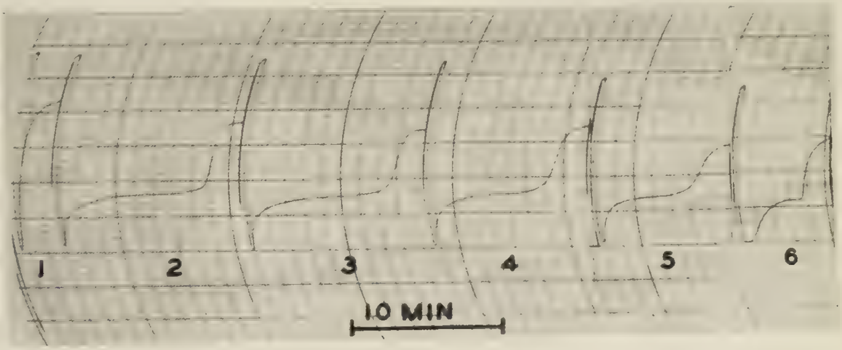


Fig. 10 The rate of hemolysis of human erythrocytes in 0.3 M glycerol in water as affected by the presence of copper (10^{-4} M), copper (10^{-4} M) plus cysteine (4.5×10^{-4} M) and butanol. 1, Normal control; 2, copper; 3, copper plus 0.01 M butanol; 4, copper plus 0.05 M butanol; 5, copper plus 0.1 M butanol; 6, copper plus cysteine plus 0.01 M butanol.

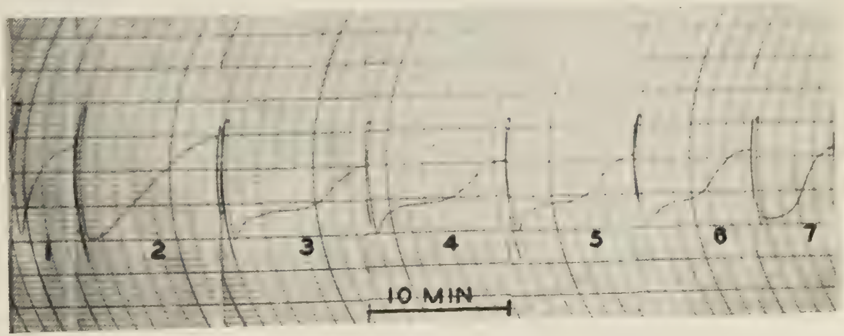


Fig. 11 The rate of hemolysis of rabbit erythrocytes in 0.3 M glycerol in water as affected by the presence of copper (2×10^{-4} M), copper (2×10^{-4} M) plus cysteine (7.5×10^{-4} M), and butanol. 1, Normal control; 2, 0.1 M butanol; 3, copper; 4, copper plus 0.01 M butanol; 5, copper plus 0.05 M butanol; 6, copper plus 0.1 M butanol; 7, copper plus cysteine.

depends on diffusion, butanol increases the permeability of erythrocytes to glycerol. Higher concentrations of butanol are more effective than lower. Both the ra of entrance and exit of glycerol are creased. These generalizations also a applicable to erythrocytes of man a rabbit, in which normal glycerol is activ transported across the cell membran when the active process is completely (almost completely) inhibited. The eff of butanol on the active transfer of gly erol across the membrane in either dir tion is inhibition. Higher concentratio of butanol are more effective than low concentrations. These generalizations a ply to normal cells and to cells in whi the active transport has been inhibit and then reversed. Butanol inhibits t active transport of glycerol in a differe way than copper, for example, for t action of the latter is reversed by a su hydryl agent, such as cysteine, while action of the former is not reversed cysteine. All of the above effects a readily and completely reversible, simp by diluting the butanol.

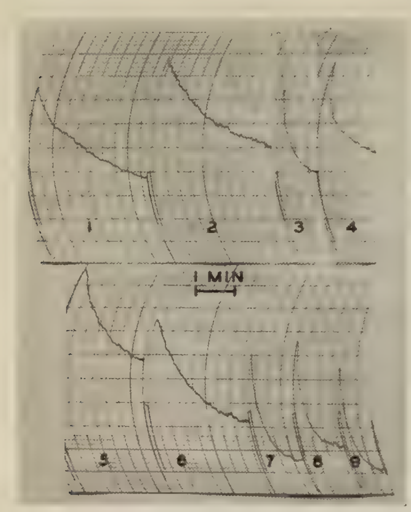


Fig. 12 The lack of effect of cysteine (1.2×10^{-3} M) on the inhibition of glycerol permeability by butanol in rabbit and human erythrocytes. Rabbit: 1, 0.1 M butanol; 2, 0.1 M butanol plus cysteine; 3, 0.01 M butanol; 4, 0.01 M butanol plus cysteine. Human: 5, 0.1 M butanol; 6, 0.1 M butanol plus cysteine; 7, 0.01 M butanol; 8, 0.01 M butanol plus cysteine; 9, control.

Thiourea. The effect of butanol on movement of thiourea across the erythr cyte membrane is much more simple a straight-forward. The rate of swelling

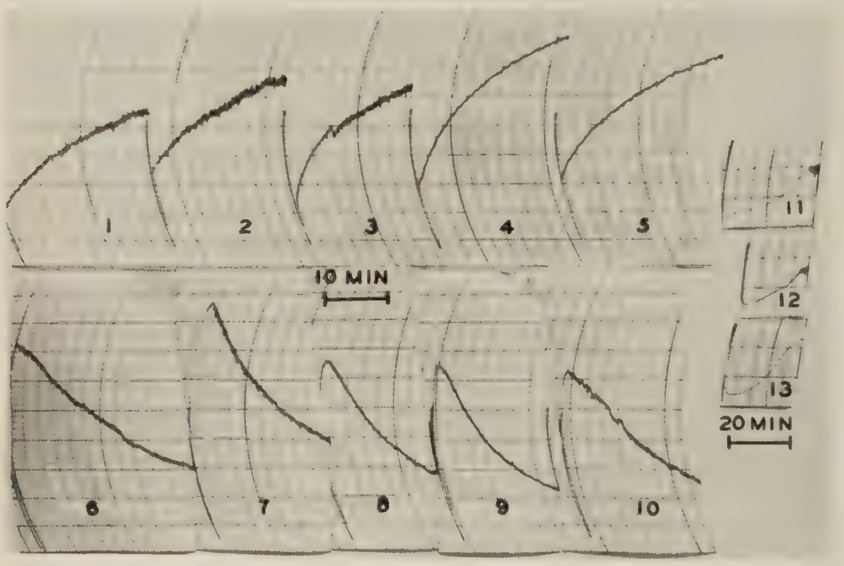


Fig. 13 The effect of butanol on the permeability of sheep erythrocytes to glycerol. 1–5, Swelling in 0.3 M glycerol in A (1, control; 2, 0.01 M butanol; 3, 0.05 M butanol; 4, 0.1 M butanol; 5, 2×10^{-4} M $CuCl_2$). 6–10, Shrinking in 1.75 × A following equilibration in 0.3 M glycerol in A (6, control; 7, 0.01 M butanol; 8, 0.05 M butanol; 9, 0.1 M butanol; 10, 10^{-4} M $CuCl_2$). 11–13, Hemolysis in 0.3 M glycerol in water (11, control; 12, 0.05 M butanol; 13, 0.1 M butanol).

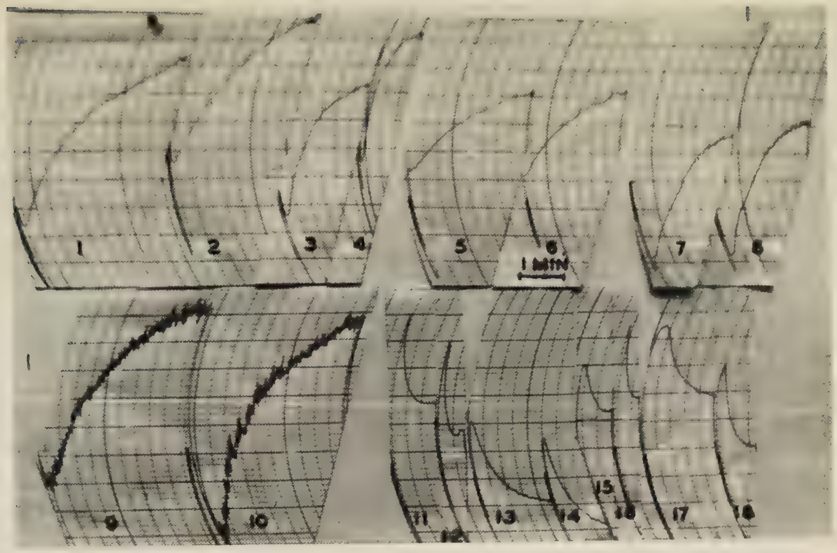


Fig. 14 The effect of butanol on the permeability of erythrocytes to thiourea. 1–10, Rate of swelling in 0.3 M thiourea in A. 1–4, Rabbit cells (1, control; 2, 0.05 M butanol; 3, 0.2 M butanol; 4, 0.4 M butanol). 5,6, Sheep cells (5, control; 6, 0.1 M butanol). 7,8, Human cells (7, control; 8, 0.1 M butanol). 9,10, Chicken cells (9, control; 10, 0.1 M butanol). 11–18, Rate of shrinking in 1.75 × A following equilibration in 0.3 M thiourea in A. 11,12, Rabbit cells (11, control; 12, 0.3 M butanol). 13,14, Sheep cells (13, control; 14, 0.2 M butanol). 15,16, Human cells (15, control; 16, 0.2 M butanol). 17,18, Chicken cells (17, control; 18, 0.3 M butanol).

shrinking of human, rabbit, sheep and chicken erythrocytes was studied and in every case the permeability to thiourea was increased by butanol. This effect was not obvious with 0.05 M butanol and less concentrated solutions but it was very obvious with 0.1, 0.2, 0.3 and 0.4 M butanol. With the exception of 0.4 M butanol (and in some cases, 0.3 M), the process was completely reversible in all cases. A few typical data are shown in figure 14.

Monoacetin. The inhibiting effect of butanol on the rate of penetration of monoacetin into rabbit erythrocytes was shown in figure 1. In figure 15 can be seen the decrease in rate of swelling of human erythrocytes in 0.3 M monoacetin in A with increasing concentrations of butanol. The accelerating effect of increasing concentrations of butanol on the rate of exit of monoacetin from sheep cells can be seen in curves 5–9. These data suggest that the effect of butanol on monoacetin permeability is similar to its effect on glycerol permeability (cf. Le Fevre, '48). Initial attempts to study the effect of copper and cysteine on monoacetin penetration indicated that it is much more difficult to demonstrate clear cut changes than in the previous study with glycerol. This would be predicted on the basis of LeFevre's data which showed only a slight effect of copper on hemolysis times in monoacetin.

NH_4Cl. Typical data obtained from studies of the effect of butanol on volume changes in M/6 NH_4Cl in A are shown in figure 16. The first 5 curves show the effect of increasing concentrations of butanol on the rate of swelling of human erythrocytes in M/6 NH_4Cl in A. It can readily be seen that the rate of swelling decreases with increasing concentration of butanol. The next three pairs of curves show the decrease in rate of swelling in the presence of butanol with rabbit, sheep and chicken cells respectively. The effect of butanol on the three types of mammalian cells was similar but with chicken cells the effect was much less noticeable. All of these changes are completely reversible with the exception of 0.3 M butanol which changes the cells irreversibly. The bottom series of curves shows the effect of buta-

nol on the rate of shrinking in 1.75 × A following equilibration in M/6 NH_4Cl in A. The first pair of curves (12, 13) are with human cells, then rabbit, sheep and chicken. To "catalyze" the rate of anion exchange, the solutions of NH_4Cl contained approximately 6 × 10^{-5} M $NaHCO_3$ (cf. Jacobs, '40). It is interesting to note that practically all of these shrink curves consist of two parts, a rapid cha at first with a subsequent slower chan The first portion is so rapid, it is diffic to say whether butanol has any effect not. In each pair of curves, however, inhibition in the presence of butanol the second slower portion of the curve

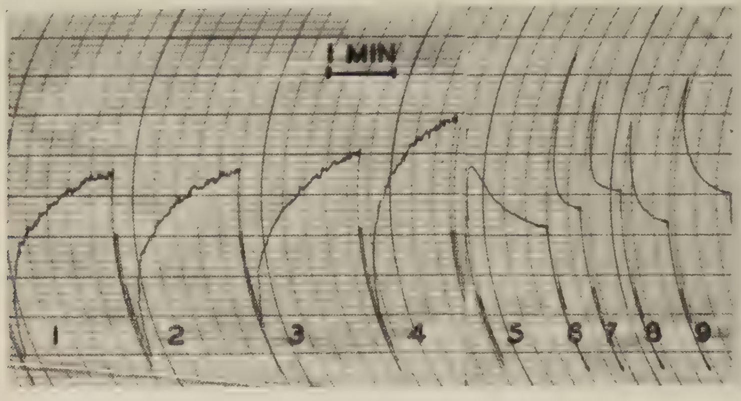


Fig. 15 The effect of butanol on the permeability of erythrocytes to monoacetin. 1–4, Swelling of human erythrocytes in 0.3 M monoacetin in A (1, 0.01 M butanol; 2, 0.05 M butanol; 3, 0.1 M butanol; 4, control). 5–9, Shrinking of sheep cells in 1.75 × A following equilibration in 0.3 M monoacetin in A (5, control; 6, 0.4 M butanol; 7, 0.3 M butanol; 8, 0.2 M butanol; 9, 0.1 M butanol).

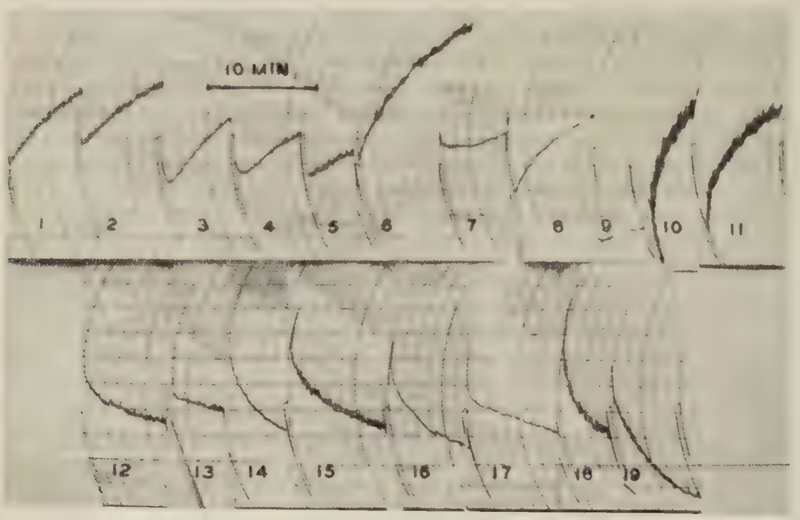


Fig. 16 The effect of butanol on the permeability of erythrocytes to NH_4Cl. 1–11, Rate of swelling in M/6 NH_4Cl in A. 1–5, Human cells (1, control; 2, 0.01 M butanol; 3, 0.05 M butanol; 4, 0.1 M butanol; 5, 0.2 M butanol). 6,7, Rabbit cells (6, control; 7, 0.3 M butanol). 8,9, Sheep cells (8, control; 9, 0.2 M butanol). 10,11, Chicken cells (10, control; 11, 0.2 M butanol). 12–19, Rate of shrinking in 1.75 × A following equilibration in M/6 NH_4Cl. 12,13, Human cells (12, control; 13, 0.2 M butanol). 14,15, Rabbit cells (14, control; 15, 0.2 M butanol). 16,17, Sheep cells (16, control; 17, 0.2 M butanol). 18,19, Chicken cells (18, control; 19, 0.1 M butanol).

adily be seen. This confirms the observaons of Jacobs and Parpart (Jacobs, '40).

DISCUSSION

The data presented indicate that butaol: (1) Increases the permeability of ythrocytes to non-electrolytes that cross e membrane by diffusion only; (2) Deeases the rate of entrance of a nonectrolyte, such as glycerol, when an acve factor is involved in its movement cross the cell membrane; and (3) Deeases the permeability of anions.

A possible explanation for these obserations can be made by assuming: (1) hat the structure of the erythrocyte memrane is similar to that suggested by Parart and Ballentine ('51); (2) That as ggested by these workers, new aqueous hannels can open up as a consequence of e movement of the lipid molecules in the embrane; (3) That normally there are termolecular forces which hold these pids in place and so close the central queous channel in each pore; and (4) hat butanol decreases these forces and so pens new, aqueous channels. Increasing oncentrations of butanol would then open the channel wider" by further derease of the forces holding the lipids in lace. In figure 4 of Parpart and Balleníne ('51) the channel is open wide enough o that a molecule of hemoglobin can ass. Various degrees of "opening" are ossible between their figures 3 and 4. Acording to the present interpretation, varis intermediate stages would be possible, ending on the concentration of butanol nd the decrease in the forces which norally keep the "swinging door closed."

With these assumptions, let us now exlain the various observations. With the door partially opened" a new aqueous hannel would exist. The higher the conentration of butanol, the "wider open" would be the "door" and the larger the queous channel. Diffusing non-electroytes could thus enter more rapidly. Also, Na and K could exchange (to maintain electrical neutrality an exchange would e necessary) by means of this channel which presumably would not be bounded y repulsive electrical charges as the normal channel is assumed to be (Parpart and Ballentine, '51, p. 147).

A close inspection of figure 4 of Parpart and Ballentine ('51) shows that as "the door opens" the size of the normal aqueous channel decreases. This would be necessary for the present explanation. It seems reasonable to assume that the active movement of molecules such as glycerol would depend on the proteins in the membrane rather than on the lipids. How else could one explain the involvement of SH groups in this transport? If, indeed, in "opening the door" the aqueous channels adjacent to the proteins decrease their size, this could explain the inhibiting effect of butanol on glycerol transport. It is true, that the diffusion of glycerol would occur more rapidly at the same time through the new aqueous channels, but this would have a negligible effect on the total rate of movement of glycerol until the active transport had been more or less completely inhibited —as in the copper experiments.

This closing of the normal aqueous channels might reasonably be expected to affect the charges which normally are present and account for the rapid penetration of anions. This change could explain the observed inhibition of anion penetration.

With this rather simple explanation of the action of butanol on the membrane of the erythrocyte, all of the observations can readily be explained.

SUMMARY

1. The effect of butanol on the permeability of human, rabbit, sheep and chicken erythrocytes to glycerol, monoacetin, thiourea and NH_4Cl has been studied.

2. In every instance where the movement of a non-electrolyte across the cell membrane depends on simple diffusion, butanol increases the permeability. This is true both for the entrance and for the exit of these molecules.

3. In those erythrocytes in which an active process is involved in the movement of a non-electrolyte across the cell membrane, butanol decreases the permeability.

4. The permeability to anions is decreased by butanol.

5. A theoretical explanation of these results is given.

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mino Acid Leakage and Amino Acid Pool Levels of ltraviolet-Irradiated Yeast Cells

PAUL A. SWENSON AND DAVID H. DOTT
Zoology Department, University of Massachusetts,
Amherst, Massachusetts[1]

One of the results of irradiating yeast ells with ultraviolet light is to cause the ss of cellular materials to the suspending edium. This effect was studied exten- ively by Loofbourow and his associates rimarily from the standpoint of the rowth-promoting activity of the leakage aterials (see Loofbourow, '49, for a re- iew). Webb and Loofbourow ('41) found or a number of members of the B vitamin omplex that their concentration is appre- iably higher in the suspending medium f irradiated compared to non-irradiated east cells. The same is true for 260 mμ bsorbing materials including nucleosides, ucleotides, and other nucleic acid deriva- ives (Loofbourow et al., '47, Thomas et l., '52 and Swenson, '60). Swenson ('60) arried out a study in which yeast cells ere uniformly labeled with P^{32} prior to radiating with ultraviolet light. With igh dosages of ultraviolet radiation, the ells leak 50% of their labeled phospho- us to the medium in a 4-hour period. he available evidence points to interfer- nce with energy-coupled cellular proces- es and to membrane damage which ermits diffusion of cellular components o the medium.

Loofbourow ('47) found amino acids mong the leakage products; tests were ade for arginine, histidine, threonine, lanine, tryptophane and tyrosine and all ere found to be present. He found no vidence for a source of these amino acids ther than cellular protein. However, a ore likely source of these amino acids s the amino acid pool. This pool, a reser- oir of free amino acids in the cell, was rst reported for yeast cells by Taylor '47). Free amino acids were found in ll of 12 strains of yeast which were xamined by Halvorson, Fry and Schwemmin ('55). The importance of the amino acid pool in the economy of the cell has been emphasized by Halvorson and Spiegelman ('53) who showed that in the synthesis of induced enzymes by yeast the pool of amino acids is used for the formation of the new protein entities.

In considering the possible role of the amino acid pool in the leakage process, several questions presented themselves. (1) Is the source of the leakage of amino acids only the pool or is there degradation of protein as well? (2) What amino acids are found among the leakage products? (3) Do all amino acids leak from the cell at the same rate? This paper deals with these questions and certain other aspects of this photobiological effect.

MATERIALS AND METHODS

The yeast used in this work was a strain of *Saccharomyces cerevisiae* originally isolated at the University of Toronto. The culture was maintained on culture slants containing 1.0% yeast extract, 2.0% glucose and 2.2% Bacto agar. Transfers were made daily. For experimental use the cells were grown in liquid culture in 500-ml flasks containing 125 ml of media. The media contained 2.0% glucose and 1.0% yeast extract. Inoculation was made by means of a sterile loop from a slant on which cells had grown for 24 hours. The flasks were shaken on a mechanical shaker for 24 hours at 30°C. Following growth, the cells were washed 4 times with distilled water and suspended in distilled water at a concentration of 2×10^8 cells ml. The experiments were carried out using water as a suspending medium for the cells because a chromatographic study of the leakage products would have been complicated by the presence of buffer salts in the concentrated leakage materials. For all experiments 20 ml of the yeast suspension were transferred to a crystallizing dish 80 mm in diameter and 40 mm high; the suspension had a depth of 4 mm.

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[1] Research supported by the U. S. Atomic Energy Commission.

Irradiation of yeast suspensions was carried out using two 14-watt General Electric germicidal lamps. These lamps were 6 mm apart and were mounted 61 mm above the level of the suspension. The germicidal lamps have 95% of their output at 2537 A; the intensity of the radiation at the center of the dish was 59.5 ergs/mm²/sec. as measured by a Hanovia ultraviolet meter. The suspension was stirred during irradiation by means of a magnetic stirrer.

Following irradiation, the samples were covered with a petri dish and incubated in an incubator-shaker maintained at 30°C. The contents of the dishes were thus shaken gently to aerate the yeast cells and prevent them from settling out. Light was excluded from the incubating samples. At intervals, 2½ ml of suspension were removed for analysis of the amino acid content of the medium and of the cellular pool. The 2½ ml of cell suspension were transferred to a 12-ml tapered centrifuge tube and the cells were packed by centrifugation. The supernatant containing the leakage products was poured off and saved for analysis.

The cells were washed once with 3 ml of distilled water and then resuspended in 2½ ml of distilled water. The pool amino acids were obtained by placing the tube in a boiling-water bath for 10 minutes (Gale, '47). After cooling, the cell residue was packed by centrifugation and the supernatant was set aside for analysis.

The level of amino acids in the leakage and pool samples was determined using the ninhydrin method which Moore and Stein ('48) described for the analysis of amino acids in effluents from ion-exchange columns. An aliquot of 0.1 ml from the leakage or pool samples was pipetted into a 16 × 150 mm test tube. To this was added 1 ml of ninhydrin solution and the solution was heated for 20 minutes in a boiling-water bath. After cooling, 5 ml of 50% n-propyl alcohol were added and
absorption at 570 mμ was read o
Bausch and Lomb Spectronic 20 Spec
photometer. The concentration of am
acids in the aliquot was obtained by in
polation on a standard curve for leuc
The results are expressed as microm
of amino acids as leucine per 4.6 mg
weight of cells. All amino acids do
react with ninhydrin to give the same
tical density at a given concentrat
Moore and Stein compared the color yi
of equimolar quantities of various am
acids to that of leucine. The relative c
yields for the amino acids found in
leakage and pool samples other than
line varies from 0.88 to 1.12. The aver
for identified amino acids exclusive of
line is 0.98. Proline reacts with ninhy
to give a yellow color with a relative c
yield of 0.05. In one experiment, c
were irradiated for 10 minutes and
isoleucine contents of pool and leak
was determined by microbiological as
(Steele et al., '40). The time-course cu
were compared to those obtained using
ninhydrin method; the two sets of cu
were in good agreement with each othe

When glucose was used as a substr
0.2 gm of reagent grade material
added to the 20 ml of yeast suspens
and solution was brought about by ra
swirling of the suspension. Zero-t
samples for pools and leaks were alw
taken before addition of the glucose.

Leakage and pool samples were
tained and processed for paper-chrom
graphic analysis as follows: Twenty m
liters of suspension were irradiated in
manner previously described and allo
to incubate for a desired length of ti
The entire suspension was then cer
fuged in a 40-ml tapered tube to pack
cells. The suspernatant was poured
and recentrifuged to throw down any c
that might have come off during deca
tion. A completely cell-free superna
was obtained. The packed cells from
first centrifuging were washed three ti
with distilled water to free them f
leakage products and were then suspen
in 5 ml of distilled water. The pool
obtained by suspending the tube with
contents in a boiling-water bath for

inutes. The pool extract was desalted sing a Reco electric desalting apparatus. lucose was removed from leakage samles by treating them with an ionxchange resin IR-120 (hydrogen form). he resin was washed free of glucose with istilled water and the adsorbed amino cids were eluted from the resin with 1 N H_4OH. The pool and leakage products ere evaporated to dryness at 80°C under acuum. The residues were then susended in 0.5 ml of distilled water. wenty-five microliters of the concentrate ere applied to the corner of a sheet of Vhatman no. 1 filter paper, 18¼ × 22 iches. Descending chromatography was mployed following the method of Benson t al. ('50) in which water-saturated pheol was used for the long dimension and utanol-propionic acid was used for the hort dimension. The dried chromatorams were sprayed with a solution of .5% ninhydrin in ethyl alcohol with a race of collidine. Identification of the mino acids was made by means of comparison with chromatograms of amino cid standards run in various combinaions, color reactions to ninhydrin and pecific chemical tests (Block et al., '58). Also employed was a method in which n unknown amino acid was eluted from n unsprayed area and applied to another aper. A known amino acid suspected to e identical to the unknown was spotted t either side and the paper was run in ne dimension in the methanol-pyridinevater solvent of Redfield ('53). If spraying revealed that the known and unknown ad the same R_f value, identification was ssumed to be established.

RESULTS

In figure 1 are graphed the time-course curves of amino acid pool levels and of the leakage of amino acids into the suspending medium for irradiated and non-irradiated endogenous cells; each process is occurring in the absence of an exogenous substrate. The amino acid pool level of non-irradiated cells increases gradually during a 6-hour period and at the same time there is a slow leakage of amino acids into the suspending medium. Irradiation of the cells for 6 minutes causes pro-

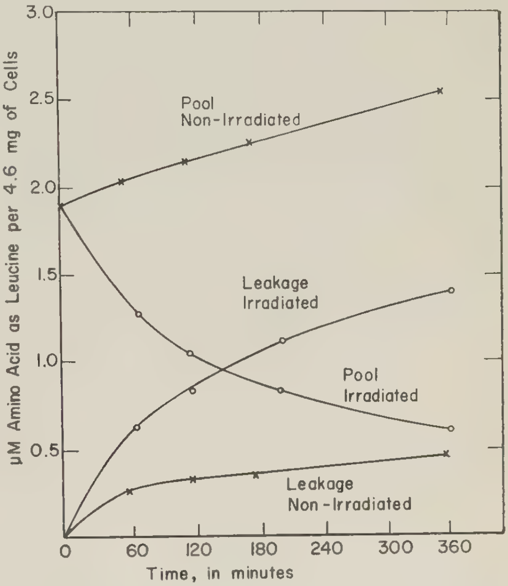

Fig. 1 Time-course curves of amino acid pools and of amino acid leakage from ultraviolet-irradiated yeast cells. Cells under endogenous conditions (no added substrate). Irradiation was for 6 minutes.

TABLE 1

Pool and leakage levels of endogenous cells irradiated with ultraviolet light (U. V.) for varying lengths of time[1]

xposure to U. V.	Time after irradiation, minutes									
	0		60		120		210		360	
	Leak	Pool	Leak	Pool	Leak	Pool	Leak	Pool	Leak	Pool
min.										
0	—	1.90	0.22	2.02	0.32	2.13	0.36	2.30	0.45	2.52
2	—	1.90	0.26	2.15	0.40	2.30	0.54	2.52	0.68	2.84
4	—	1.90	0.33	1.81	0.61	1.80	0.90	1.85	1.02	2.00
6	—	1.90	0.55	1.33	0.81	1.05	1.10	0.80	1.36	0.58
8	—	1.90	1.10	0.82	1.40	0.40	1.82	0.10	2.60	0.00
10	—	1.90	1.42	0.54	2.00	0.18	2.60	0.00	3.06	0.00

[1] The data are expressed as µmoles of amino acids as leucine per 4.6 mg of cells.

nounced changes. The pool level is reduced to about one-third of its original value and this reduction is accompanied by rapid leakage of amino acids into the medium. The data presented graphically in figure 1 are tabulated in table 1 together with data obtained from experiments for which cell suspensions were irradiated for 2, 4, 6, 8, and 10 minutes. With increasing dosages of ultraviolet radiations leakage is progressively more extensive, and except for samples irradiated for two minutes, the pool levels fall with increasing rapidity. For the 8- and 10-minute irradiated samples the pool levels are reduced to zero at 210 and 360 minutes respectively. The amount of leakage does not simply reflect the loss of amino acids from the pool. Figure 2 is a bar-graph plot of the pool and leakage levels of irradiated cells 360 minutes after irradiation. The total number of free amino acids is the sum of those in the pool and in the medium. For each dosage the bar has a pool and leakage component and the total free amino acids is given by the height of the bar. For the purpose of comparison
zero-time pool level is represented b
separate bar at the extreme left of
figure. The non-irradiated cells after
minutes show a 55% increase in total
amino acids over the zero-time level. L
age from cells which were irradiated
two minutes is 50% greater than
from non-irradiated cells. The pool l
does not decrease with such an irradia
dosage; instead it increases by 50% (
the pool level at zero time. This is appr
mately 15% greater than that of the r
irradiated control. As a result of irr
ating the cells with the relatively r
dosage of two minutes, the total numbe
free amino acids exceeds by 17% thos
the non-irradiated cells after the sa
period of incubation. Larger dosages ca
further decreases in pool levels and
creases in the amount of leakage but v
4 minutes of irradiation the final p
level, although lower than that of eit
the non-irradiated or the two-minute
radiated cells, is approximately equal
the zero-time pool level. Furtherm
fewer total free amino acids are accour
for in cells irradiated for 4 and 6 min
than those irradiated for two minu
The total free amino acid level is a
minimum with 6 minutes of irradiat
and for this dosage the total is appr
mately equal to that of the amino a
pool at zero time. Increasing the irra
tion dosage to 8 and 10 minutes cau
further increases in the total number
free amino acids, all of which are leak
products, the pool level having fallen
zero.

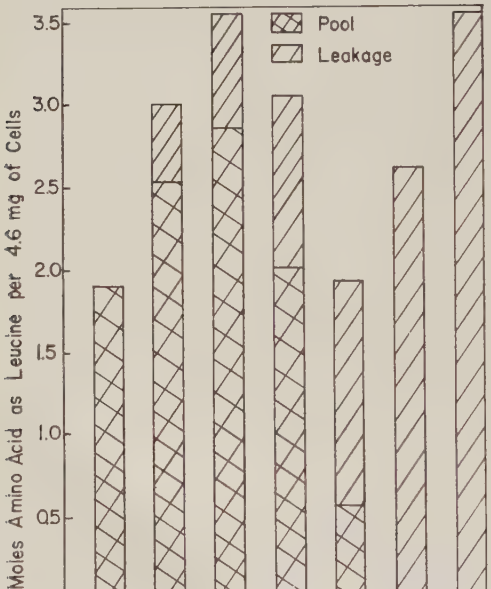

Fig. 2 Bar-graph comparing amino acid pool and leakage levels 6 hours after irradiation for different lengths of time. Endogenous cells were employed (no added substrate).

Halvorson, Fry and Schwemmin ('
have reported that yeast cells incuba
with glucose in a nitrogen-free medi
undergo an amino acid pool depleti
Such depletion is shown in figure 3 wh
the pool is seen to drop to about 40%
its original level after two hours and to
main stabilized at that level. Leak
however, is seen to be negligible from c
metabolizing glucose. The results of
radiating cells for 8 minutes immedia
after the addition of glucose are also s
in figure 3. The pool level falls rapidl
a level far below that of the non-irradia
cells and after 6 hours is almost at z

akage is likewise very rapid and exten-ve. Figure 4 compares the leakage and ool curves of the glucose-irradiated cells with those of irradiated cells having no exogenous substrate. Both samples were irradiated for 8 minutes. The initial rates of both processes are greatest with the glucose-irradiated cells but after 6 hours the extent of damage, as indicated by final leakage and pool levels, is approximately the same for both. The fact that the oxidation of glucose by irradiated cells influences the rate of leakage suggests that if irradiation were accomplished before the addition of glucose, the leakage and pool patterns might be further altered. Figure 5 compares the results of irradiating two cell suspensions for 8 minutes, but differing in the time at which glucose was added relative to the time at which each of the suspensions was irradiated. When glucose is added immediately after irradiation is completed, leakage is more extensive and rapid than if irradiation is begun at the same time that glucose is added to the cell suspension. The pool curves are essentially the same for both sets of experimental conditions.

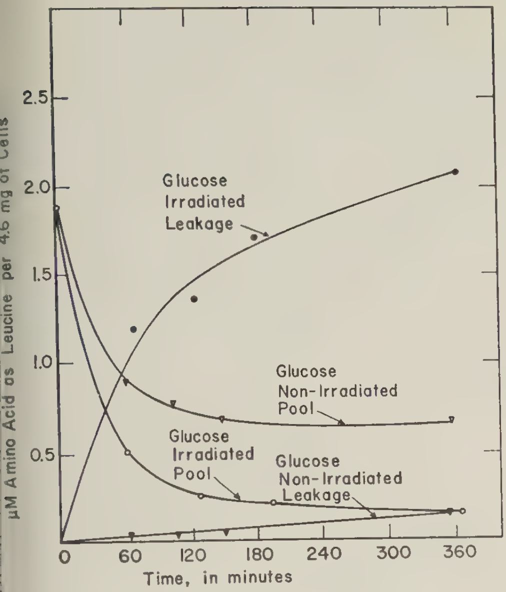

Fig. 3 Pool and leakage time-course curves r cells irradiated with glucose present. Irradiition was for 8 minutes. Glucose was added and rradiation started at zero time.

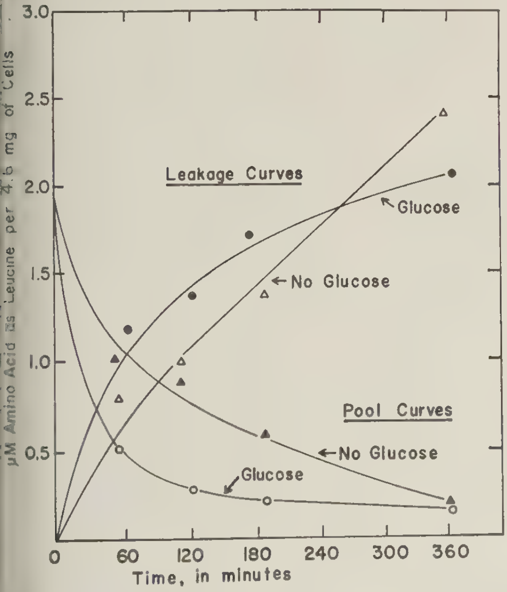

Fig. 4 A comparison of the pool and leakage urves of irradiated cells with and without gluose. Glucose was added and irradiation started t zero time; irradiation was for 8 minutes.

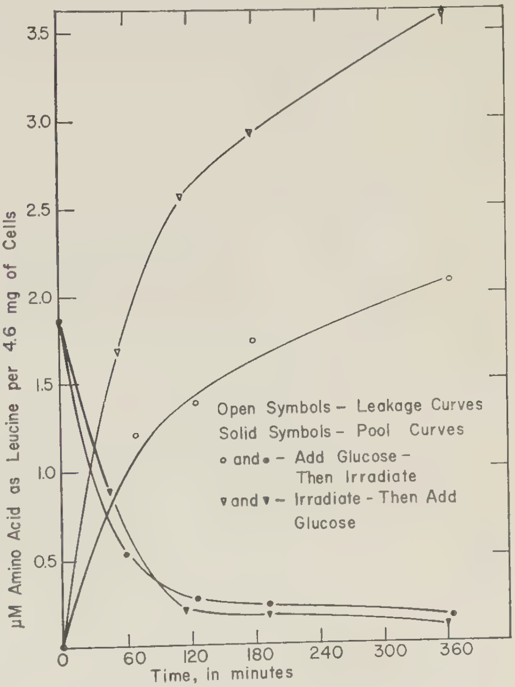

Fig. 5 The influence of the time at which glucose is added to irradiated cells on the amino acid pool and the leakage of amino acids. Irradiation was for 8 minutes.

The influence of glucose metabolism on the depletion of amino acid pools of non-irradiated cells to a lower, stabilized level after two hours of incubation raises an interesting question. What effect do ultraviolet radiations have on the stabilized pool? The following experiment was designed to answer this question. Four samples of yeast were suspended with glucose in the usual manner and all were incubated for a period of two hours, the time at which the intermediate pool level is reached. At this point the samples were all washed two times with distilled water. The washing was necessary because, in the presence of glucose, nucleic acid derivatives, as indicated by the absorption of ultraviolet light of wave-length 260 mμ, leak into the medium. These 260-mμ-absorbing materials would act as a filter to protect the cells from the ultraviolet radiations. Following washing, all were resuspended in distilled water. No glucose was added to two of the samples; one of these was irradiated, the other was not. The remaining two samples received glucose; one of these was irradiated and the other was not. In each case where samples were irradiated they were exposed to the radiations for 8 minutes. The results are seen in figure 6. As expected, the pool level remains constant in the non-irradiated control cells which receive glucose. The non-irradiated cells incubated without glucose undergo pool replenishments and after three hours the pool level is approximately 80% of the original level. The results with the irradiated cells are of considerable interest in that the pool levels do not fall. Those irradiated and incubated with glucose exhibit an increase in pool level during the first two hours but after another hour the level is the same as that of the non-irradiated control. The cells irradiated and incubated without glucose undergo pool replenishment to the same extent as their non-irradiated counterparts.

The leakage curves for the experimental conditions described above are also seen in figure 6. The loss of amino acids from glucose-irradiated cells is approximately the same as that from irradiated cells without glucose. Leakage in both cases is much less extensive than t from cells irradiated with the same dos of ultraviolet radiation at zero time. the other hand, the rates of leakage fr non-irradiated cells with and without g cose are approximately the same as fr similarly-treated cells which are not s jected to the two-hour pretreatment w glucose.

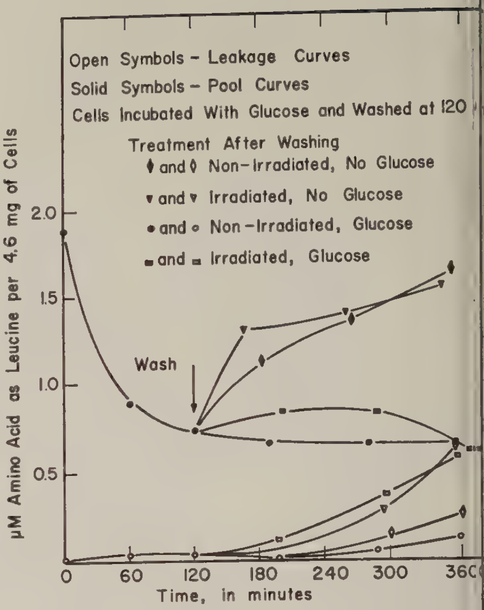

Fig. 6 Pool replenishment and leakage f irradiated and non-irradiated pool-depleted c incubated in the presence and absence of cose. Irradiation was for 8 minutes.

The pool curves of figure 6 suggest t the metabolizing of glucose promotes binding of amino acids in some way so to prevent the pool level from falling lowing irradiation of the cells with ul violet light. Suppose it is assumed t the binding process in glucose-metabo ing, non-irradiated cells proceeds a faster rate than that of pool depletion. cordingly, it would be predicted that c with partially depleted pools, when radiated, would maintain their pools a high level and would leak fewer am acids than those which are irradiated the time that glucose is added. Figur shows the results of such an experime Cells were allowed to undergo pool de tion for 7 minutes in the presence of cose before irradiating them for 8 minu

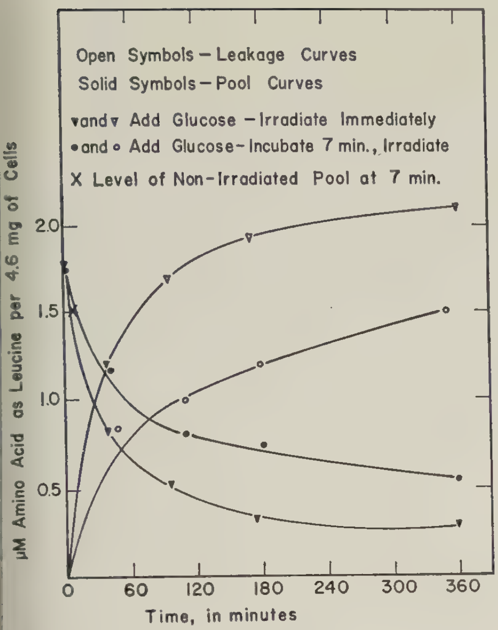

Fig. 7 The effect of ultraviolet radiations on glucose-metabolizing cells with complete and partially-depleted pools. Irradiation was for 8 minutes.

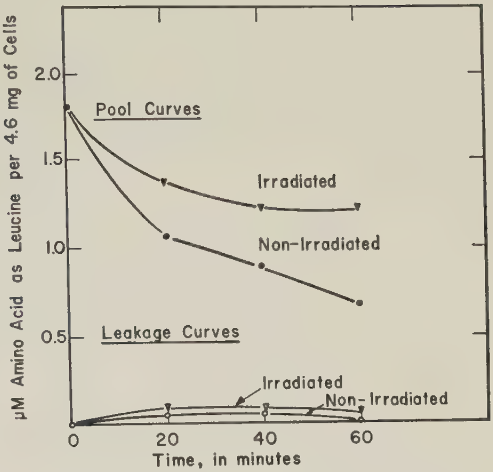

Fig. 8 The effect of ultraviolet radiations on the utilization of the amino acid pool. Irradiation was for 2½ minutes.

During the 7-minute period the pool level was depleted to approximately 93% of its original value. For the control experiment irradiation was begun immediately after the addition of glucose. Referring still to figure 7, it is seen that the falling off of the pool level as well as leakage is considerably less for the cells irradiated at 7 minutes than for those irradiated at zero time. Pool depletion in non-irradiated glucose-metabolizing cells must represent a synthetic process. Because synthetic processes are known to be sensitive to ultraviolet radiations, it should be found that glucose-stimulated pool depletion is inhibited by these radiations. A relatively mild dosage, 2½ minutes of irradiation, was used to test this hypothesis. Such a dosage was selected to avoid leakage and falling of the pool level, both of which occur following higher irradiation dosages. Figure 8 compares the leakage and pool curves of non-irradiated and 2½-minute irradiated cells. Leakage is at the same low rate from each. Pool depletion, hence synthesis, is apparently inhibited by the comparatively mild irradiation treatment as evidenced by the fact that the pool level of the irradiated cells after two hours is higher than that of the non-irradiated control.

The experimental results reported in the previous sections have dealt with overall changes in the pool and leakage levels. It is of considerable interest to compare the composition of (1) the amino acid pool in freshly-harvested cells, (2) the pools of cells that have been irradiated, and (3) the leakage products from irradiated cells. Such comparisons would allow an interpretation as to whether or not the leakage of amino acids from yeast cells is a selective process, that is, a process in which individual amino acids are lost to the medium from the cells at different rates. Figure 9 is a scale reproduction of a paper chromatogram of the amino acid pool of freshly-harvested cells. Seventeen distinct areas are seen which include probably 19 amino acids or amino acid derivatives. Unknown C is not found in the pools of freshly-harvested cells and its significance as a dotted area will be explained presently.

It will be recalled from figure 1 that in the absence of an exogenous substrate the amino acid pool gradually increases and amino acids slowly leak from the cells. Chromatograms were made of pool and leakage samples obtained from such cells

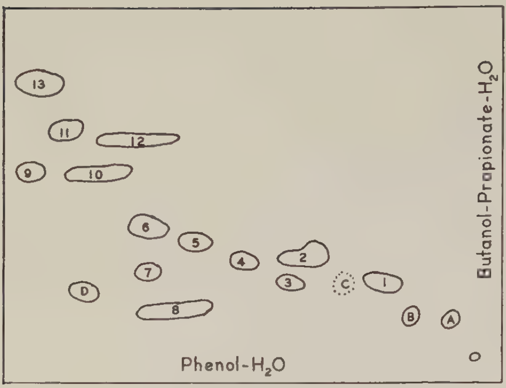

Fig. 9 Scale reproduction of a chromatogram of the free amino acid pool of resting cells and of leakage amino acids from irradiated cells incubated for 6 hours. Chromatograms for both are alike except for component marked C; this appears only in the pools of irradiated cells. The numbers refer to the following components: (1) aspartic acid, (2) glutamic acid, (3) serine, (4) glycine, (5) threonine, (6) alanine, (7) glutamine, (8) lysine-arginine, (9) proline, (10) tyrosine, (11) valine, (12) phenylalanine, (13) leucine-isoleucine, A, B, C, and D unknowns. O indicates origin.

at hourly intervals. No qualitative cha in the pool composition were found only traces of aspartic acid, glutamic a serine, glycine, alanine, arginine and sine were found among the leakage terials.

The composition of the amino acid and leakage products following irradia was studied first for cells under end nous conditions, that is, in the absenc an exogenous substrate such as gluc Six minutes of ultraviolet radiations w used for these experiments; after 6 ho all of the pool amino acids are seen on chromatogram of the leakage produ (fig. 9). Table 3 gives the overall levels and indicates the presence or sence of individual amino acids in leaks and pools at hourly intervals up 6 hours following irradiation. For the age employed the final pool level is duced to 25% of its original value and number of leakage amino acids is appro mately 85% of the number in the ze time pool. The pool data provide evide

TABLE 2

The amino acid composition[1] *of the pools and leakage products of endogenous irradiated cell indicated by paper chromatography*[2]

Amino acid	Zero-time pool	Hours after irradiation 1 P	1 L	2 P	2 L	3 P	3 L	4 P	4 L	5 P	5 L	6 P
Aspartic acid	+	+	+	+	+	+	+	+	+	+	+	+
Glutamic acid	+	++	+	+	+	+	+	+	++	+	++	+
Serine	+	+	+	+	+	+	+	+	+	±	+	—
Glycine	+	+	+	+	+	+	+	±	+	±	+	+
Threonine	+	+	+	+	+	+	+	+	+	+	+	±
Alanine	+	+	+	+	+	+	+	+	+	+	+	±
Glutamine	+	+	+	+	+	—	+	—	+	—	+	—
Lysine and arginine	++	++	++	++	++	+	++	+	++	+	++	+
Leucine and isoleucine	+	+	+	±	+	±	+	±	+	±	+	±
Valine	+	+	+	—	+	—	+	—	+	—	+	—
Proline	+	+	+	±	+	—	+	—	+	—	+	—
Tyrosine	+	++	±	+	+	±	+	±	+	±	++	—
Phenylalanine	+	++	+	++	+	+	+	+	++	±	++	±
Unknown A	+	+	+	+	+	+	+	+	+	+	+	+
Unknown B	+	—	+	—	+	—	+	—	+	—	+	—
Unknown C	—	+	—	+	—	±	—	±	—	±	—	±
Unknown D	+	+	+	+	+	+	+	+	+	+	+	+
μM amino acids per 4.6 mg of cells	1.90	1.18	0.62	0.75	1.10	0.65	1.25	0.60	1.50	0.60	1.60	0.50

[1] Key: P, pool; L, leak; +, present; ++, relatively large amount; —, absent; ±, trace.
[2] Irradiation time was 7 minutes.

TABLE 3

The composition of the amino acid pool for cells incubated with glucose[1]

Amino acid	Zero-time pool	Hours after incubation with glucose					
		1	2	3	4	5	6
Aspartic acid	+	+	+	+	+	+	+
Glutamic acid	+	++	++	+	+	+	++
Serine	+	+	+	+	+	−	−
Glycine	+	+	+	+	+	−	−
Threonine	+	+	+	+	+	+	+
Alanine	+	+	+	+	+	+	+
Glutamine	+	+	+	+	−	±	±
Lysine and arginine	++	++	++	++	++	++	+
Leucine and isoleucine	+	+	+	±	±	±	±
Valine	+	±	+	±	±	±	±
Proline	+	−	−	−	−	−	−
Tyrosine	+	±	±	±	−	±	−
Phenylalanine	+	+	+	±	±	±	±
Unknown A	+	+	+	+	+	+	+
Unknown B	+	−	−	−	−	−	−
Unknown C	−	−	−	−	−	−	−
Unknown D	+	+	+	+	+	+	+
μM amino acids per 4.6 mg cells	1.80	0.90	0.79	0.70	0.70	0.68	0.71

[1] Key: +, present; −, absent; ±, trace; ++, relatively large amount.

that all amino acids do not leak from the pool at the same rate. For example, unknown B is absent from the pool after one hour, glutamine, proline and valine after three hours, and tyrosine and serine after 6 hours. Phenylalanine and threonine are present in the pool in trace amounts after 6 hours while aspartic acid, glutamic acid, glycine, unknown A and unknown D are still present in considerable quantities after 6 hours. Reference has already been made to unknown C. This substance appears in the pool one hour after irradiation but curiously is never found among the leakage products. All of the resting pool components are found among the leakage products one hour after irradiation.

Before examination of the results of irradiating cells with glucose present, it is desirable to see what changes take place in the amino acid pool which undergoes depletion in non-irradiated cells as a result of glucose metabolism. In table 3 it is seen that proline and unknown B are absent from the pool after one hour, serine and glycine after 5 hours and tyrosine after 6 hours. Several other amino acids, phenylalanine, valine, glutamine, leucine and isoleucine are found as only traces at various times after incubation. Only a small amount of leakage takes place from these cells and only traces of glutamic acid, threonine, glutamine, leucine, isoleucine and phenylalanine are found on any of the leakage chromatograms.

The results of irradiating cells for 6 minutes in the presence of glucose, presented in table 4, show that although the leakage level is high after one hour, some amino acids are not among the leakage products. Proline, tyrosine, phenylalanine and unknown B are not seen in the leaks at any time during the 6-hour period. Only traces of valine, phenylalanine, unknown D, leucine and isoleucine are found after one hour. The leakage complement is predominantly aspartic acid, glutamic acid, serine, glycine, threonine, alanine, glutamine, unknown A and one or both of lysine and arginine. The pool level is reduced rapidly with 6 minutes of irradiation and proline, tyrosine and unknown B are absent after one hour; these three pool components, therefore, are not found in either the pools or the leaks at any time after one hour. The various amino acids disappear from the pool at different times, but after 6 hours good spots are found only for aspartic acid, glutamic acid and serine. Unknown C, a component of

TABLE 4

The amino acid composition of pools and leakage products of cells irradiated with glucose pres

Amino acid	Zero-time pool	Hours after irradiation										
		1		2		3		4		5		6
		P	L	P	L	P	L	P	L	P	L	P
Aspartic acid	+	+	+	+	+	+	+	+	+	+	+	+
Glutamic acid	+	+	+	+	+	+	++	+	++	+	++	+
Serine	+	+	+	+	+	+	+	±	+	—	+	+
Glycine	+	+	+	+	+	+	+	—	+	—	+	—
Threonine	+	+	+	+	+	+	+	±	+	±	+	±
Alanine	+	+	+	+	+	+	+	+	+	±	+	±
Glutamine	+	+	+	+	+	+	+	+	+	±	+	±
Lysine and arginine	++	+	±	+	±	+	±	+	+	±	+	±
Leucine and isoleucine	+	±	—	±	—	±	±	±	±	±	±	±
Valine	+	±	—	±	—	±	±	±	±	±	±	±
Proline	+	—	—	—	—	—	—	—	—	—	—	—
Tyrosine	+	—	—	—	—	—	—	—	—	—	—	—
Phenylalanine	+	±	—	±	—	±	—	±	—	—	—	—
Unknown A	+	+	+	+	+	+	+	+	+	+	+	—
Unknown B	+	—	—	—	—	—	—	—	—	—	—	—
Unknown C	—	—	—	—	—	—	—	—	—	—	—	—
Unknown D	+	±	—	±	±	±	±	—	±	—	±	—
μM amino acids per 4.6 mg cells	1.85	0.40	1.20	0.20	1.45	0.15	1.40	0.10	1.80	0.10	1.90	0.10

[1] Glucose was added and irradiation begun at zero time; irradiation time was 7 minutes. Key: +, present; ++, relatively large amount; —, absent; ±, trace.

the pool of irradiated cells incubated without a substrate, is not found in the pool of glucose-irradiated cells.

DISCUSSION

Amino acids are leaked to the medium at a slow rate from non-irradiated endogenous yeast cells. Loofbourow ('47) reported that for ultraviolet-irradiated and non-irradiated cells the ratio of amino nitrogen in the medium was 14 to 1. The amino acids which leak out cannot be moved back into the cell because that involves an active transport system requiring an exogenous energy source such as glucose (Taylor, '47). C^{14}-labeled aspartic acid is rapidly taken up by yeast cells when glucose is being burned, but if glucose is not present only a negligible amount of radioactivity is found inside the cells after 4 hours of incubation (Swenson, unpublished data). In the case of phosphate, it has been shown by Swenson ('60) that P^{32} leaks from uniformly-labeled, water-suspended endogenous yeast cells at a negligible rate. Perhaps phosphates are transported back into the cell when they leak out. In contrast to amino acids, orthophosphate is taken up by endogen cells at an appreciable rate, although rate is much slower than when gluc is present (Swenson, '58).

The increase of the amino acid level in the absence of an exogenous ergy source suggests that a restriction the energy supply of the cell results the labile cellular proteins being bro down to form free amino acids. T mechanism of internal pool replenishm was proposed by Halvorson, Fry, Schwemmin ('55) to explain replen ment for pool-depleted cells; Halvor ('58) provided convincing proof that source of the replenished amino acid protein.

The addition of glucose to a yeast pension provides energy for the ac transport across the cell membrane of amino acids which leak to the exter Hence, net leakage of amino acids fr these metabolically active cells is slight. Glucose also provides energy the synthesis of protein as is indicated the disappearance of a large portion of amino acid pool. It might be expec that no amino acids would be miss

om the pool until after two hours, the
ne at which the net number of amino
ids in the pool becomes constant. The
sults of the chromatographic analyses
the pool reveal that proline and un-
own B are missing after one hour and
at other amino acids do not disappear
til after 5 hours. The nature of the
otein formed during pool depletion is
t known but it is important to note that
alvorson ('58) has shown that during
is period the internal replenishment
ocess is taking place concurrently.

Some interesting contrasts to the non-
radiated amino acid pool and leakage
cture in yeast have been described for
her microbial organisms. Mandelstam
58), for example, found that for *E. coli*
mino acids leak approximately 5 times
ster from cells without glucose than
om those having the exogenous sub-
rate, but the leak-level for the cells with-
t glucose is so extensive that it exceeds
e zero-time pool level. Furthermore, the
ol level increases by 30% over a 90-
inute period when glucose is present.
vidently, in *E. coli* the free amino acid
ol cannot be mobilized for protein syn-
esis to the same extent as in yeast.
hen glucose is absent the pool level
rops by 15% during the 90-minute incu-
ation period. This drop does not reflect
ynthesis as in the case of the glucose-
cubated yeast cells but rather it reflects
e extensive loss of amino acids from
e cell.

Gale ('47) tested for the diffusion of
lutamic acid and lysine from *Streptococ-
us faecalis*. Glutamic acid does not leak
rom cells suspended in salt solution, but
hen an energy source such as glucose is
resent the pool level falls and glutamic
cid leaves the cells. The situation is
uch more complex for lysine (Najjar
nd Gale, '50).

Holden and Holman ('59) studied the
ptake of glutamic acid in *Lactobacillus
rabinosus*. Cells which have accumu-
ated glutamic acid excrete glutamate
fter they are washed; the loss is in-
reased when glucose and other amino
cids are in the suspending medium.

Recently, Chesbro and Evans ('60) re-
orted that amino acids leak from resting cell suspensions of *Streptococcus faecium* when the suspending medium is maintained at a pH above 8.5. The rate of loss of amino acids is a function of the logarithm of the pH and the process can be partially reversed by the addition of glucose. The influence of pH has not been tested on leakage of amino acids from non-irradiated yeast cells but at pH values below 2.5 and above 8.5 phosphate compounds leak extensively (Swenson, '60).

Ultraviolet radiations modify the leakage and pool relationships for the yeast cell. A mechanism which comes immediately to mind to explain the leakage of amino acids is damage to the cell membrane. If the total number of free amino acids inside and outside the cell were to remain constant following irradiation, the problem of the explanation of leakage would be greatly simplified. However, the number present varies greatly with the irradiation dose. Following two minutes of irradiation the total number of amino acids exceeds that of the control. This is the consequence of an increase in the number of amino acids in the pool and a slight increase in leakage. It is postulated that the radiations accelerate the rate of endogenous breakdown of protein and also cause some damage to the cell membrane. A less likely possibility is that in endogenous cells there is a protein synthesis process at work and that this process is inhibited by ultraviolet radiations. It is possible that the energy derived from the burning of endogenous reserves could be used for protein synthesis, but if any synthesis does take place its rate must be exceeded by the rate of protein degradation in order for the total number of free amino acids to increase.

At high dosages where the pool level is completely exhausted and the leakage level exceeds the total of pool and leakage amino acids of endogenous cells, the rate of protein degradation might again be accelerated and membrane damage allow all of the pool amino acids to leak out of the cell. It is the intermediate dosages that present the greatest problems of interpretation because, although the leakage levels gradually increase with the dosage, the total number of amino acids present is either equal to or less than that of the control.

Undoubtedly, the radiations cause multiple effects in the organism. The result of the interplay of a number of processes is being measured; some of these processes are accelerated and others are inhibited. It may be that at low dosages the radiations accelerate the breakdown of cellular proteins; this would account for the increase in total amino acids. At intermediate dosages the normal endogenous breakdown of proteins might be inhibited and at high dosages the breakdown might once more be accelerated but for a different reason. The nature of these processes and how they are accelerated or inhibited by ultraviolet radiations is a problem which at the moment cannot be resolved.

It has been shown by Swenson ('60) that leakage of phosphate compounds from irradiated yeast cells metabolizing glucose is 5 times that from irradiated endogenous cells; in this work glucose was added after the cells were irradiated and no experiment was performed in which the glucose was added at the time irradiation was begun. In the case of amino acids, it has been shown in the present paper that the rate and extent of the leakage are greater when the sugar is added to the cells upon completion of the irradiation process than if glucose is added at the time irradiation is begun. It seems clear that ultraviolet radiations favor the degradation of cellular proteins and that when glucose is present the rate is accelerated. The significance of the extra amino acids found in the medium of the cells irradiated prior to the addition of glucose will be discussed in greater detail in a later paragraph.

The depletion of the free amino acid pool upon incubation with glucose has already been discussed. Halvorson, Fry, and Schwemmin ('55) reported that pool replenishment could be brought about by restricting the energy source of the cells and irradiating them with low dosages of ultraviolet light. These workers washed the cells free of glucose after different incubation times and then carried out the irradiation; in each case the pool level of glutamic acid was increased. The res of Halvorson ('58) and those reporte the present paper show that pool rep ishment may be brought about me by washing the cells free of nutrie Replenishment can again be expla in terms of energy available to the for protein synthesis (Halvorson, ' upon withdrawal of the glucose, synth ceases, labile protein is degraded and a result, additional amino acids appea the pool. Again, in the absence of an e enous energy source, no active trans system is in operation to move the am acids back into the cell. The dosage ultraviolet light used in the present w to irradiate the pool-depleted cells is which, for freshly-harvested endogen cells, causes all the pool amino acids t lost to the medium. Some leakage f the pool-depleted irradiated cells is but what is more important, after drastic treatment the pool level rem constant for cells reincubated in gluc and pool replenishment takes place the cells without glucose. As has alre been suggested in the section on resu the pool amino acids may be bound some way, chemically or physically, that they do not leak readily through damaged membrane. The pool-replen ing amino acids in the irradiated c without glucose are probably bound i similar manner and it must be postula that ultraviolet radiations under these cumstances do not interfere with the b ing process. The leakage which occ may be the result of the radiations, w the replenishing amino acids arise f protein that is degraded as a result of normal replenishment process.

The experiment in which cells w irradiated at the time of addition of cose and 7 minutes after the addition glucose (fig. 7) has a bearing on discussion of the influence of glucose the rate and extent of leakage as well on the hypothesis concerning the bind of amino acids during glucose meta lism. The results of this experiment sh that leakage is least extensive and pool levels remain highest in the c which have been incubated for 7 minu before irradiation. One possible inter

ion of these results is the following.
e conversion of some of the pool amino
ids to proteins or protein precursors dur-
g the 7 minutes leaves fewer amino
ids to leak out of the cells. An objection
this reasoning is that the difference
tween the amounts leaked from the two
adiated samples is nearly twice the
nount which disappears from the pool
ring the 7 minutes of incubation. Fur-
ermore, even with fewer amino acids
esent in the pool at 7 minutes, the pool
ould be reduced to the same level as
e control. An alternate proposal is that
me amino acids are mobilized for pro-
n synthesis, thereby removing them
om the boiling-water extractable pool,
d other amino acids are bound as pre-
ously suggested, resulting in their being
nsiderably less sensitive to the ultra-
olet radiations. The radiations prevent
rther binding and the remaining acids
e susceptible to leakage from the cells.

The same line of reasoning can be ap-
ied to the situation where cells are
radiated prior to addition of glucose. The
diations prevent binding of amino acids
d this inhibition, coupled with accel-
ated degradation of proteins and mem-
ane damage, causes faster and more
tensive leakage than from cells for
hich irradiation begins at the time glu-
se is added. In the latter case the bind-
g of some amino acids may occur during
e first few minutes of irradiation and
is may account for the lack of a greater
fference between the leakage levels of
ese cells and endogenous cells irradiated
ith the same dosage. Studies have not
en made to determine the conditions
quired to cause the amino acids to re-
rn to the radiation-sensitive state which
isted prior to incubation with glucose.
resumably if glucose were withheld for
long enough period this would take
lace.

It is difficult to speculate concerning
e nature of the binding of amino acids
hich has been proposed to explain the
sults presented in this paper. There is,
fact, some question as to whether any
f the amino acids in the pools of various
lls are free or whether they are bound
Gale, '53 and Christensen, '55). The possibility also exists that amino acids can be physically compartmentalized in a separate pool within the cell. Cowie and McLure ('59) have found two separate pools for *Candida utilis*. The concentrating pool is found only when exogenous amino acids are present; these amino acids enter the expandable pool and may be shifted to the internal pool which serves as a direct source of amino acids for protein synthesis. Similar results were reported for *Neurospora crassa* by Zalokar ('61) who has suggested that the internal pool could be the water compartment of the liquid cytoplasm and that the amino acids of the expandable pool are adsorbed on cellular components or confined to a liquid compartment such as the endoplasmic reticulum. If separate pools are involved in the system under consideration in the present paper, the pools differ from those of *Candida* and *Neurospora* in that exogenous amino acids are not involved in their formation. It may be, however, that the amino acid pool present in freshly harvested endogenous cells is, in fact, the expandable pool formed during growth and that the presence of glucose shifts amino acids into an internal pool which is resistant to the effects of radiation.

Ultraviolet radiations inhibit the formation of induced enzymes (Swenson and Giese, '50) and the uptake of phosphate (Swenson, '58) in yeast cells. In the light of these facts, the inhibitory effects on the utilization of the amino acid pool for protein synthesis were predicted and a low dosage of irradiation was employed so that leakage would not be a factor. The pool level does remain higher than that of the control, indicating that synthesis is inhibited. However, an objection to this experiment being accepted as evidence of proof of inhibition is the fact that the same low dosage for endogenous cells causes an increase in the amino acid pool level. Much more critical experiments are required before a definite statement can be made.

The foregoing discussion of the results has emphasized that the action of ultraviolet radiations in causing leakage of amino acids from yeast cells cannot be explained in terms of membrane damage alone. There are metabolic factors as well that influence the rate and extent of leakage. The chromatographic analysis of the leakage products and pool components of irradiated cells contributes further evidence to support the idea that membrane damage alone does not explain leakage. All amino acids do not leak from the cell at the same rate and one component of the irradiated cell pool, unknown C, appears in the pool one hour after irradiation and does not leak out. The leakage of amino acids provoked by ultraviolet light is, therefore, like the leakage of phosphate, (Swenson, '60) a selective process.

SUMMARY

1. A study was made of the effect of ultraviolet radiations on leakage of amino acids from yeast cells and on the amino acid pools of these cells.

2. Amino acids leak slowly from non-irradiated endogenous cells and the amino acid pool level of these cells increases slowly over a 6-hour period.

3. Ultraviolet radiations cause additional leakage of amino acids. Leakage increases with dosage, and except for low dosages which cause the pool to increase in size, the amino acid pool falls below its original level. With sufficiently high dosages the pool falls to zero within 3½ hours after irradiation and the amino acids leaking out exceed the total free amino acids, leakage plus pool, of the non-irradiated cells. This indicates that cell protein, as well as the amino acid pool, is a source of the leakage amino acids.

4. Glucose accelerates the rate of leakage and the rate at which the pool drops in irradiated cells. Leakage is most rapid and extensive when glucose is added after irradiation is complete.

5. Irradiation of cells that have undergone pool depletion by pre-incubation for two hours with glucose does not result in a further drop of the amino acid pool. The pool level of cells irradiated and reincubated with glucose present remain essentially constant; if glucose is absent after irradiation, pool replenishment takes place to the same extent as for non-irradiated cells.

6. Irradiation of cells for which the is partially depleted by a 7-minute in bation with glucose results in less leak and a less extensive pool drop than cells with complete pools at the time cose is added and irradiation begun. I postulated that glucose influences leak because the increase in metabolism fa the physical or chemical binding of am acids of the pool, a state in which they relatively insensitve to the ultravi radiations.

7. A chromatographic analysis of amino acid pool and leakage products made before and at hourly intervals a irradiation. All of the members of amino acid pool leak out of the cell; so disappear completely from the pool others do not. One unidentified ninhyd positive substance appears only in pool of irradiated cells.

8. The significance of the above fi ings is discussed. Leakage of amino a from yeast cells following ultraviolet r ation is concluded to be a selective proc influenced by the metabolic activity of cell. Membrane damage probably occ but it alone cannot account for the served facts.

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he Nature of Impulses of Single Tarsal Chemo- eceptors in the Butterfly, *Vanessa indica*[1]

KIMIHISA TAKEDA[2]
Department of Biology, Faculty of Science, Kyushu University, Fukuoka, Japan

In the blowfly Hodgson and Roeder 56) have shown in single chemosensory irs the spikes from two chemosensory eurons; one neuron responding to sugars d the other to non-sugars. Wolbarsht d Dethier ('58) have demonstrated in e same chemosensory hairs the spikes om a mechanoreceptor. The generator tential of the chemoreceptor (Morita, 9) with hyperpolarization by $CaCl_2$ or her inhibitors (Morita and Yamashita, 9) has also been shown in the blowfly.

In the butterfly Morita, Doira, Takeda d Kuwabara ('57) reported several kinds spikes in the chemosensory hairs dur- g application of sucrose and/or NaCl. owever, in their experimental arrange- ent, the effect of electrical current flow- g through the chemosensory neuron and imulation of the mechanoreceptor in- lved in the hair were not controlled. The esent report demonstrates the nature of iefly the chemoreceptor activities ob- ined under improved experimental con- itions, which showed a different mode om those in the blowfly.

MATERIALS AND METHODS

The materials are the same as used in e previous experiments (Morita et al., 7; Morita and Takeda, '59), the tar- l chemosensory hairs of the butterfly, *anessa indica*. The experiments were per- rmed in winter with the room tempera- re ranging from 10 to 13°C. The prep- rations survived for several hours and e reproducible results were obtained.

The chemosensory hairs are distributed isymmetrically in a row on each of ven- ral (or ventrolateral) and lateral sides of ll 5 tarsomeres of the hind and mid legs. hey are also seen on dorsal side. A few hemosensory hairs are grouped at the distal end of each tarsomere. A slightly different designation from that of Morita et al. ('57) is used here, i.e., the ventrolateral hair in the previous paper correspond to the lateral hair in this paper.

A depression of approximately 1 μ both in diameter and in depth is observed at the tip of the hair, where the receptor site of chemosensory neuron exists (Dethier and Wolbarsht, '56). The distal process of the neuron is observed to extend to the depression.

The method used to pick up the electrical activity of the chemosensory hair was basically the same as that of Hodgson and Roeder ('56). A leg of the butterfly was amputated at the proximal part of tibia. A platinum wire inserted into the cut end was used as the indifferent electrode. A glass capillary placed over the tip of the hair served for chemical stimulation and also as the recording active electrode.

A high input impedance, cathode-follower preamplifier (Tomita and Torihama, '56) which had a grid current of the order of 10^{-12} A in direct coupled condition was used. A selected ceramic capacitor of 5 pF was inserted between the active electrode and the grid of the preamplifier. Thus the effect of electrical current on the chemosensory neuron was minimized (cf. Wolbarsht, '58).

Ion exchanged distilled water was used to dissolve stimulating chemicals except for non-electrolyte stimulants, which were dissolved in a conducting solution, i.e., 10 mM NaCl in most cases. This concen-

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[2] Present Address: Department of Physiology, Tokyo Medical and Dental University, Yushima, Tokyo, Japan.

tration of NaCl seemed not to affect the stimulating action of the non-electrolytes used. The sugars used, including inositol, were guaranteed reagents, and were freshly dissolved usually in 10 mM NaCl before experiment, which for brevity were described in the figures and tables using only the name and concentration of the sugars.

The chemosensory hair was rinsed with 2^{-3} M NaCl after each stimulation. The intervals between stimulations varied from 5 to 20 minutes, depending on the experiments. An interval of 5 minutes was enough for disadaptation after ordinary stimulation (fig. 9a, sucrose responses), although sometimes an after effect remained after repeated applications of inhibitory stimuli (table 2, lowest three cases). Mechanical stimulation was given by bending the chemosensory hair with the stimulating capillary.

Although in most cases the recording was made until about the 7th second of stimulation, photographic records show the responses during approximately the second and third second unless otherwise stated, except for the cases of mechanical stimulation. The first second may be important in the sensory response, however, technical difficulties prevented us from using the early part of it. However, our results correlate well with the strength of stimulus as will be shown. In all records, a downward deflection represents an increase in positivity at the active electrode, and one sweep a 0.25 sec. duration. At the bottom of each record 1 mv calibrating
pulses with slow decline to the base
are shown. Impulse frequencies are
resented by the number of spikes per
ond.

RESULTS AND DISCUSSION

Mechanoreceptor

Two kinds of spikes, the large (1 t
mv) and the small (usually less tha
mv) spikes could be discriminated in
gle chemosensory hairs throughout
present experiments. The small spi
showed no correlation with any chemi
stimulus used, but they clearly respon
to intentional mechanical stimulation (
1). Therefore these small spikes have b
identified as from the mechanorecep
By applying conc. H_2SO_4 to the hair
train of the large spikes appeared, wh
gradually deteriorated and disappea
within 10 or more seconds. This may
an injury discharge of the chemosens
neuron. The large spikes never appea
regardless of the sort of the chemi
stimuli after the H_2SO_4 application. T
large spikes are thus originating in
chemoreceptor as will be shown later. T
small spikes were still elicited by mecha
cal stimulation following the H_2SO_4 ap
cation (figs. 1F; 2).

Although the mechanoreceptor spi
responded well at the moment of bend
(fig. 2), they occasionally appeared d
ing ordinary chemical stimulation (fig
small spikes) because during which co
plete absence in deformation of the H
was impossible, so that care should

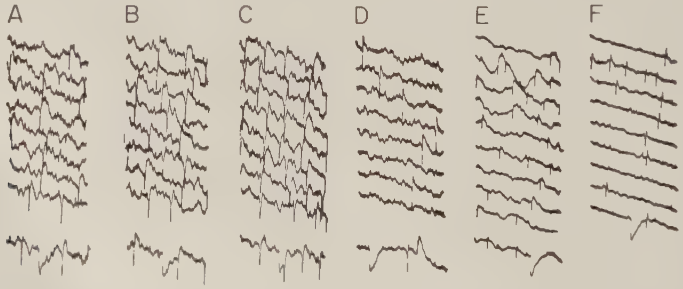

Fig. 1 Chemoreceptor (large) and mechanoreceptor (small) spikes. Lateral hair. Responses: A, to 2^{-2} M sucrose in 2^{-3} M NaCl; B, to 2^{-1} M sucrose in 2^{-3} M NaCl; C, to 1 M sucrose in 2^{-3} M NaCl; D, to 1 M NaCl; E, to bending the hair with the capillary used in D; F, to bending after exposing the hair tip to conc. H_2SO_4. *Recording was made in alphabetical order on a single hair in each of all photographic records.*

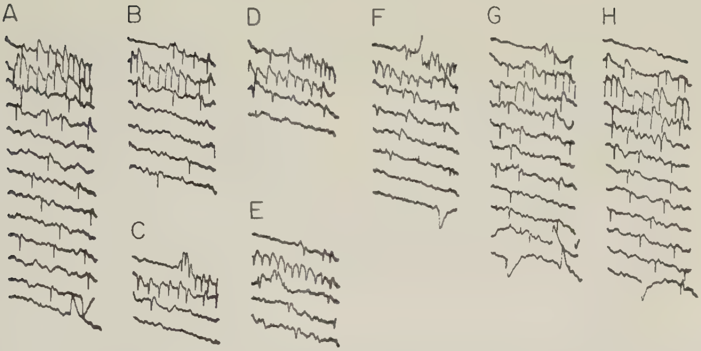

Fig. 2 Change in form of the single mechanoreceptor spikes during repetitive stimulations. Lateral hair. A–F, responses to repetitive bending (several records were omitted between each record); G and H, responses after a rest of 15 minutes.

ken to identify them in studying the emoreceptor response. They showed a aracteristic feature of mechanorecep- r spikes; a change in spike height with ange in spike frequency (fig. 2) as re- rted previously (Wolbarsht and Dethier, 8; Morita and Takeda, '59). However, e mechanoreceptor spikes often showed comparable duration to that of chemo- eptor spikes (fig. 1) in contrast to the evious finding that the former having longer time course allowing it to be dis- nguished from the latter (Morita and keda, '59). This apparent difference ll be explained by figure 2, in which the echanoreceptor spikes show a decrease height and a prolonged duration fol- wing repeated mechanical stimulation. ey have recovered after a rest of 15 inutes.

The relative height of chemo- (large) d mechanoreceptor (small) spikes was nsistent in the ventral, lateral and dor- l hairs. The mechanoreceptor spikes corded occasionally exceeded 1 mv in e lateral hairs, although only at high equencies. However, as they were al- ays less than 1 mv in the ventral hairs d easily distinguished from the chemo- ceptor spikes, the ventral hairs were used ost often for the study of the chemo- ceptor.

Sugar receptor

A train of the large spikes which oc- rred specifically in response to sucrose owing reasonable correlation with the ncentration, especially during the early part of the stimulation was identified as the activity of a single sugar receptor (fig. 3). The sugar receptors were widely distributed in most ventral hairs although no pattern of distribution was observed. They were also located in lateral hairs.

Adaptation. The impulse frequency of a single sugar receptor in response to sucrose declines during the first 10 or more seconds of stimulation and reaches a more or less steady level (fig. 4). The frequency for the first second was estimated based on a linear extrapolation from the record of the last three-fourths of the first second.

The impulse frequency of the *Vanessa* sugar receptor is quite regular in contrast to the irregularity reported by Hodgson and Roeder ('56) in the blowfly. The sugar receptor in *Vanessa* discharged impulses of a fairly high frequency (at highest more than 80/sec. which showed usual adaptation), and responded to 2^{-7} M sucrose (table 1). They were always represented by the large spikes so far examined, which seems to be a general property in *Vanessa*.

In one case the impulse frequency increased during the first several seconds for successive sucrose stimulations, although the sugar receptor adapted as usual to the other sugars (table 2). However, this butterfly had been starved for approximately 10 days before use, which may have caused this unusual behavior.

Response to NaCl. The sugar receptor did not respond to NaCl at all in approximately 50% of the total hairs examined

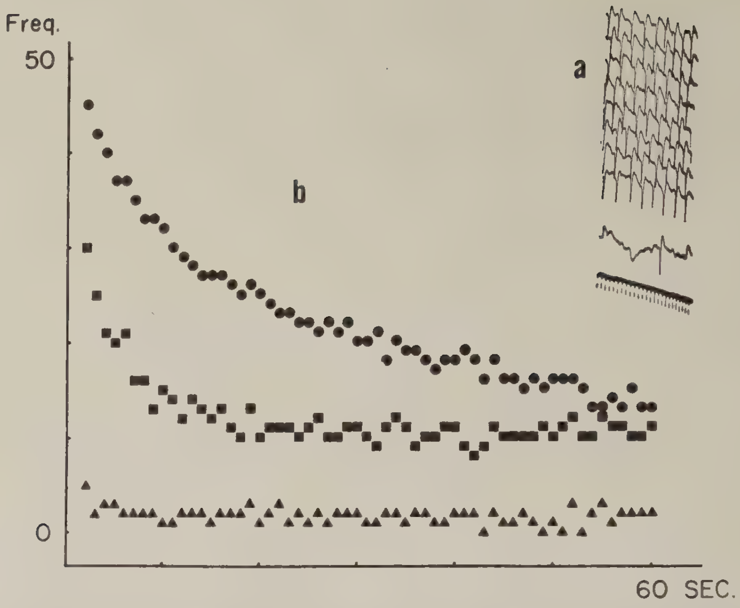

Fig. 3 Responses of a sugar receptor to ascending concentration of sucrose. a, The spikes in response to 2^{-1} M sucrose; time mark, 10 msec. b, Impulse frequencies: ▲, to 2^{-5} M; ■, to 2^{-3} M; and ●, to 2^{-1} M sucrose. (Intervals between stimulations ca. 20 minutes.)

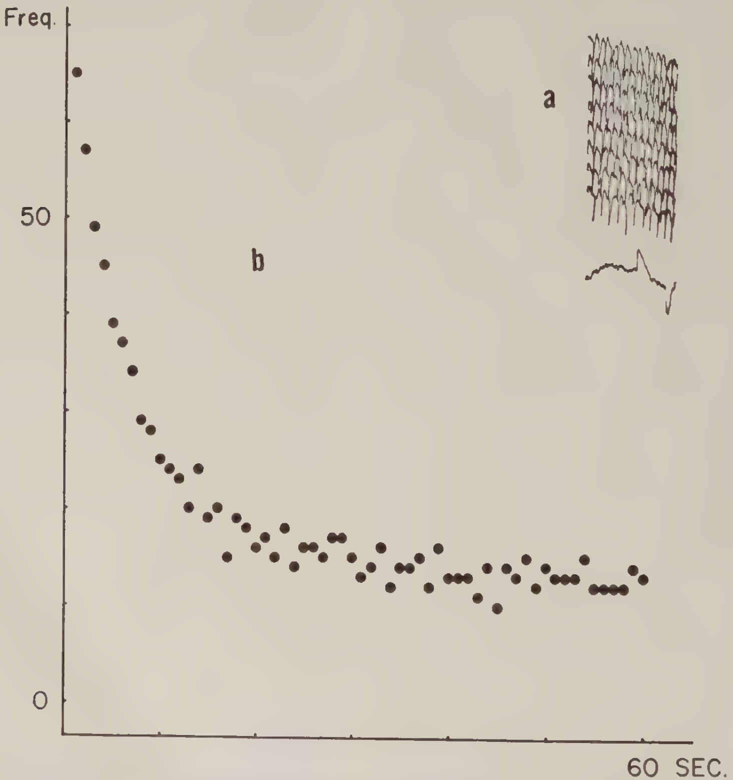

Fig. 4 Response of a sugar receptor to 2^{-2} M sucrose. a, The spikes. b, Adaptation of impulse frequency.

g. 5). In other hairs the large spikes, ich were apparently the same in their rm as those of the sugar receptor, ap- ared in very low frequencies (one spike r several seconds) to NaCl. The fre- ency was a little higher in some hairs t no correlation with concentration of aCl was found (fig. 7C, I). Since the rge spikes could not be correlated with e concentration of any chemical stimuli ed except for sugars, they are regarded the spikes of sugar receptor. Thus the sponsiveness of the sugar receptor to aCl is, if at all present, remarkably low comparison with that to sucrose.

Response to qualities. The sugar re- ptor discharged no impulses on applica- n of 2^{-2} N acetic acid, 1 mM quinine drochloride in 10 mM NaCl, or 2^{-2} M gCl$_2$ (fig. 6), even when the receptor icited the spikes by NaCl (fig. 7).

A mixture of 2 mM soluble saccharine, hich was effective concentration to a man (Warren and Pfaffmann, '59), and mM NaCl did not fire a sugar receptor which was not also fired by NaCl (fig. 6C). It did, however, fire those sugar receptors which were also fired by NaCl but only to the extent as by pure NaCl (fig. 7B, I). Therefore soluble saccharine seems to have no effect on the sugar receptor, in contrast to depressing effect of quinine hydrochloride (fig. 7D).

Depression by NaCl. Sodium chloride in the stimulating sucrose solution generally depressed the frequency of the sugar receptor impulses. This is a characteristic which can be used to distinguish sugar receptor impulses from those of the sugar-NaCl receptor. This depression can well be correlated with the concentration of mixed NaCl (fig. 8). It appeared with fairly low concentration (i.e., 2^{-4} M) of NaCl, and was so marked that the response to 2^{-2} M sucrose was almost completely abolished by 1 M NaCl. This could not be explained solely by the physicochemical nature of stimulating solution (cf. Wolbarsht, '58), so that a direct in-

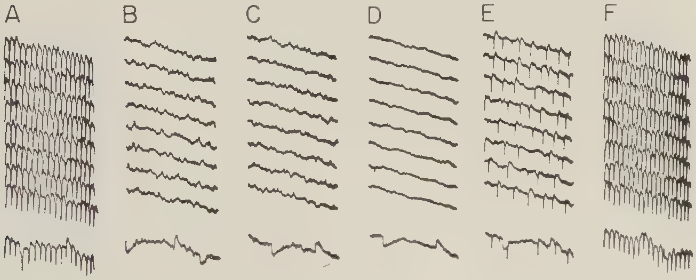

Fig. 5 Responses of a sugar receptor to sucrose and NaCl. Responses: A and F, to 2^{-2} M sucrose; B, to 2^{-6} M NaCl; C, to 2^{-4} M NaCl; D, to 2^{-2} M NaCl; E, to 2^{-2} M sucrose in 2^{-2} M NaCl. (Intervals ca. 5 minutes.)

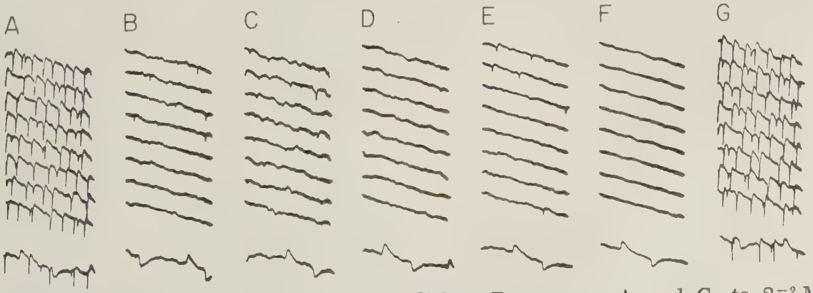

Fig. 6 Responses of a sugar receptor to qualities. Responses: A and G, to 2^{-2} M sucrose; B, to 2^{-3} M NaCl; C, to 2 mM soluble saccharine in 5 mM NaCl; D, to 2^{-2} N acetic acid; E, to 1 mM quinine hydrochloride in 10 mM NaCl; F, to 2^{-2} M MgCl$_2$. (Intervals ca. 8 minutes.)

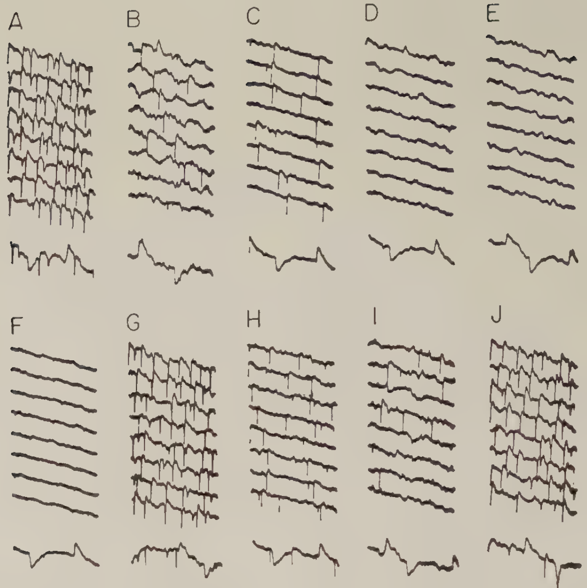

Fig. 7 Responses of a sugar receptor to qualities. Responses: A, G and J, to 2^{-2} M sucrose; B, to 2 mM soluble saccharine in 5 mM NaCl; C, to 2^{-3} M NaCl; D, to 1 mM quinine hydrochloride in 10 mM NaCl; E, to 2^{-2} N acetic acid; F, to 2^{-2} M $MgCl_2$; H, to 2^{-2} M sucrose in 2^{-2} M NaCl; I, to 10 mM NaCl. (Intervals ca. 8 minutes.)

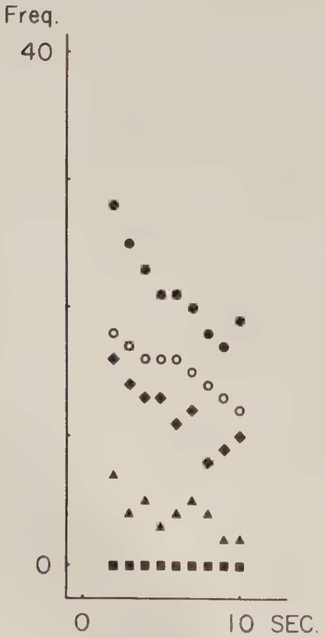

Fig. 8 Depression of impulse frequency of a sugar receptor by NaCl. Responses in the order of applications to 2^{-2} M sucrose mixed: ●, in 2^{-6} M NaCl; ♦, in 2^{-4} M NaCl; ▲, in 2^{-2} M NaCl; ■, in 1 M NaCl; ○, in 2^{-6} M NaCl. (Intervals ca. 20 minutes.)

hibitory action of NaCl on the sugar
ceptor is assumed.

Hodgson ('57) reported an appr
mately inverse relationship between
quencies of the S and L spikes in
blowfly, which he interpreted as an in
action between the two receptors.
Vanessa Morita and Takeda ('59)
ported the depression of sucrose respo
with conc. NaCl, however, those spi
which had been regarded as in respons
sucrose were the small spikes (about
mv) and not those large spikes (2 m
With reference to the present work,
possibility that those small spikes are fr
mechanoreceptor remains. The pres
depression by NaCl, a monovalent sal
interesting since the same depression
a divalent salt has been reported in
blowfly (Evans, '58).

Responses to sugars. The stimulat
effectiveness of various sugars on sin
sugar receptors was examined from
point of view of their configurations.
sugar receptors did not respond to lact
D-galactose, inositol, or L-sorbose but
sponded to D-glucose, more strongly
D-fructose and most strongly to sucr

TABLE 1

Impulse frequencies of a sugar receptor to sugars (intervals ca. 10 min.). Recording was made in the order of top to bottom in all tables.

Stimulant	Impulse frequency				
	Time after stimulus applied (sec.)				
	2	3	4	5	6
2^{-1} M lactose	0	0	0	0	0
2^{-1} M D-galactose	0	0	0	0	0
2^{-1} M inositol	0	0	0	0	0
2^{-1} M L-sorbose	0	0	0	0	0
2^{-1} M D-glucose	7	3	3	3	3
2^{-1} M sucrose	36	31	27	25	23
2^{-1} M D-maltose[1]					
2^{-1} M D-fructose	10	10	6	7	5
2^{-6} M sucrose	5	2	1	2	2
2^{-5} M sucrose	6	3	2	0	4
2^{-4} M sucrose	10	9	7	5	7
2^{-3} M sucrose	16	13	10	9	8
2^{-2} M sucrose	19	16	12	13	9
2^{-1} M sucrose	27	26	24	21	18
10 mM NaCl	0	0	0	0	0
2^{-7} M sucrose	2	4	0	0	—

[1] Impulse frequency was not counted because of the obscurity of spikes.

ese were all applied as 2^{-1} M solution
10 mM NaCl (table 1).
Two pairs of monosaccharides which
ve similar configurations; D-galactose
d D-glucose, L-sorbose and D-fructose
ere applied in 1 M concentration, and the
gar receptors did not respond to either
galactose or L-sorbose, but did respond
both of the other sugars.
In order to compare the stimulating
fectiveness between D-glucose and D-fruc-
se unit as monosaccharide and as the
nstituent of sucrose, the responses to
^{1} M D-glucose, 2^{-1} M D-fructose, a mix-
re of 2^{-2} M D-glucose and 2^{-2} M D-fruc-
se, and 2^{-2} M sucrose were examined.
e response was always strongest to the
crose and weakest to the D-glucose
ig. 9a; table 2). The relative magnitudes
the responses to the D-fructose and to
e mixture of D-glucose and D-fructose
ffered by the cases; in a case similar
ig. 9a), in other cases greater to the
rmer (first comparison in table 2) or
eater to the latter (second comparison
table 2). Referring to the time after
ssolving the sugars in these cases, these
fferences could be explained by the pro-
rtions of α- and β-forms of these mono-
ccharides in the solution which change
th time.

It is noticeable in figure 9a that the adaptation of the sugar receptor to D-fructose or the mixture of D-glucose and

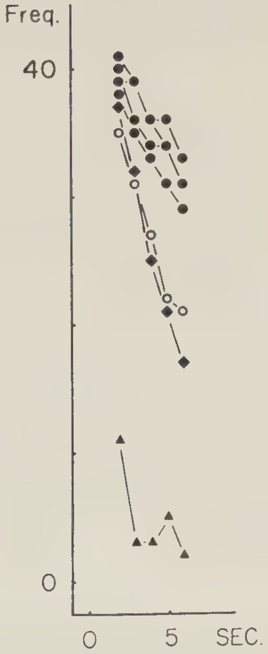

Fig. 9a Impulse frequencies of a sugar receptor to sugars. (From fig. 9b.) Responses: ●, to 2^{-2} M sucrose; ▲, to 2^{-1} M D-glucose; ◆, to 2^{-1} M D-fructose; ○, to the mixture of 2^{-2} M D-glucose and 2^{-2} M D-fructose.

D-fructose is more rapid than that to sucrose, which is statistically significant. The difference in adaptation between these monosaccharides and sucrose is more prominent in table 2.

The responses of sugar receptors to disaccharides; trehalose, D-maltose, turanose were compared with those to crose because of their similarity in figurations. The sugar receptors did

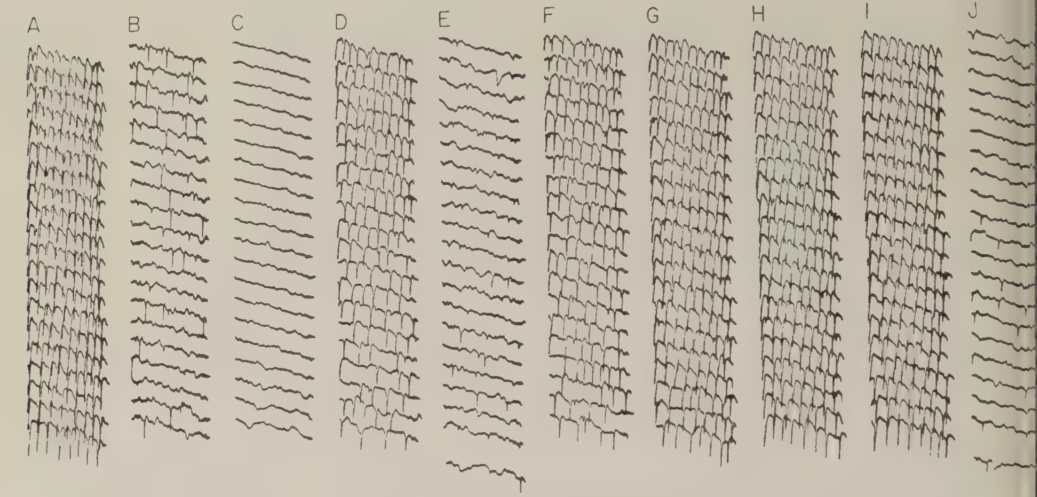

Fig. 9b Responses of a sugar receptor to sugars (from 2nd to 6th second of stimulation). Responses: A and G-I, to 2^{-2} M sucrose; B, 2^{-1} M D-glucose; C, to 2^{-1} M trehalose; D, to 2^{-1} M D-fructose; E, to 2^{-1} M turanose; F, to the mixture of 2^{-2} M D-glucose and 2^{-2} M D-fructose; J, to 2^{-1} M D-maltose. (Intervals ca. 6 minutes.)

TABLE 2

Impulse frequencies of a sugar receptor to sugars and qualities (from fig. 10)

Stimulant	Impulse frequency					
	Time after stimulus applied (sec.)					
	2	3	4	5	6	7
2^{-2} M sucrose	66	74	78	84	86	87
2^{-1} M D-maltose	8	10	8	8	6	11
2^{-1} M trehalose	0	0	0	0	0	0
2^{-1} M turanose	22	22	23	23	20	20
2^{-3} M NaCl	3	0	0	0	0	0
2^{-2} M sucrose	61	66	67	72	73	—
2^{-2} M sucrose	58	60	65	69	69	72
2^{-2} M D-glucose	14	13	12	11	11	10
2^{-1} M D-glucose	18	16	16	15	14	12
2^{-2} M D-fructose	20	19	18	17	15	15
2^{-1} M D-fructose	28	26	25	23	22	22
2^{-2} M D-glucose·2^{-2} M D-fructose	23	22	20	20	18	16
2^{-2} M sucrose	47	52	52	53	52	55
2^{-1} M D-glucose	10	8	8	9	6	6
2^{-1} M D-fructose	19	16	14	12	14	—
2^{-2} M D-glucose·2^{-2} M D-fructose	21	21	18	18	17	—
2^{-2} M sucrose	48	48	51	51	52	53
2 mM soluble saccharine·5 mM NaCl	1	0	0	0	0	0
1 mM quinine hydrochloride·10 mM NaCl	0	0	0	0	0	0
2^{-2} N acetic acid	0	0	0	0	0	0
2^{-2} M $MgCl_2$	0	0	0	0	0	0
2^{-2} M sucrose	34	35	36	37	37	—
2^{-2} M sucrose	37	38	40	44	44	—
2^{-2} M sucrose	41	44	46	48	48	48

pond to 2^{-2} M or 2^{-1} M trehalose (figs. : C; 10C), but D-maltose or turanose cited sugar receptor spikes, which were nerally deformed and smaller than those response to sucrose (figs. 9b: E, J; B, D). These spikes were thus rather ficult to identify at the beginning of the periment. However, the mechanorecep- : spikes were much smaller and they uld be easily distinguished from these formed ones (fig. 10B, D). The sugar ceptors occasionally responded to D-mal- se or turanose after a silent period or by increase in impulse frequency (fig. 9b: J).

The silent period and shape of the spikes ggest that D-maltose and turanose may ve an inhibitory effect in addition to causing stimulation, in spite of the fact that each of their monosaccharide constituents alone being a stimulant. In connection with this idea, it is interesting that repeated applications of repellents caused similar reductions in spike size, which was shown during the following sucrose stimulations to have recovered with impulse frequency (fig. 10 V–X).

The impulse frequency in response to turanose was usually lower than to D-glucose, and lower to D-maltose than to turanose.

The above results on mono- and disaccharides indicate that the D-glucose or D-fructose unit, especially the latter, is effective in sugar stimulation. Furthermore sucrose, which has been shown to be the

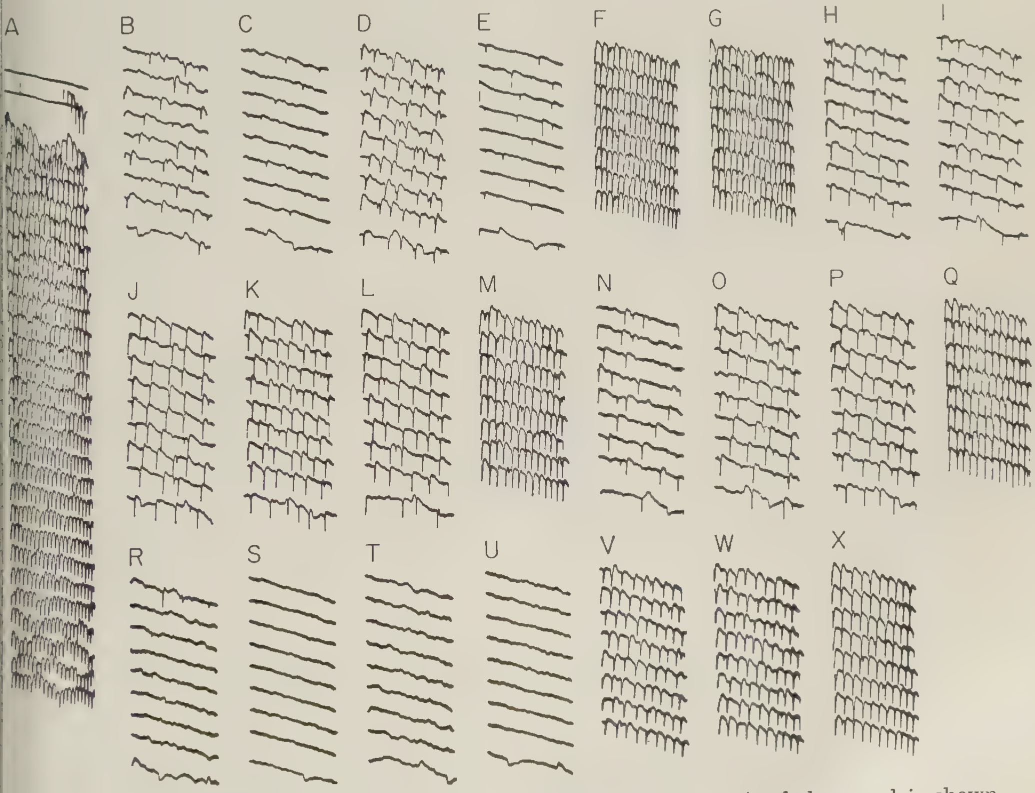

Fig. 10 Responses of a sugar receptor to sugars and qualities. A whole record is shown in A. In A, deflection of the baselines in the lower part is due to calibrating pulses. Responses: A, F, G, M, Q and V–X, to 2^{-2} M sucrose; B, to 2^{-1} M D-maltose; C, to 2^{-1} M trehalose; D, to 2^{-1} M turanose; E, to 2^{-3} M NaCl; H, to 2^{-2} M D-glucose; I and N, to 2^{-1} M D-glucose; J, to 2^{-2} M D-fructose; K and O, to 2^{-1} M D-fructose; L and P, to the mixture of 2^{-2} M D-glucose and 2^{-2} M D-fructose; R, to 2 mM soluble saccharine in 5 mM NaCl; S, to 1 mM quinine hydrochloride in 10 mM NaCl; T, to 2^{-2} N acetic acid; U, to 2^{-2} M $MgCl_2$. (Intervals ca. 10 minutes.)

strongest stimulant to these sugar receptors is suggested to have some structural validity besides a simple sum of its monosaccharide constitutents.

Our results in *Vanessa* agree with those in rats (Hagstrom and Pfaffmann, '59) in that the relative neural response is sucrose > glucose > maltose. However, the sugar receptors in *Vanessa* did not respond to inositol at all which was the exceptional stimulant among polyhydroxy compounds in the behavioral tests in the blowfly (Dethier, '55). Also they did not respond to L-sorbose which in the blowfly had nearly the same threshold as D-glucose.

Sugar-NaCl receptor

In some ventral hairs, large spikes occurred in response to NaCl as well as to sucrose (fig. 11). This type of receptor responsible for those spikes will be referred to as a sugar-NaCl receptor. The sugar-NaCl receptor responded with rather similar impulse frequencies to 2^{-2} M sucrose, 2^{-4} M NaCl, 2^{-2} M NaCl, and 1 M NaCl, but with a little lower frequency to 2^{-6} M NaCl which is statistically significant, i.e., the impulse frequency saturated at a fairly low concentration of NaCl (table 3). The tendency that the adaptation of impulse frequency to NaCl was more rapid than to sucrose was observed (tables 3, 4).

The sugar-NaCl receptor responded to 2^{-2} M sucrose, 2^{-2} M NaCl, and a mixture of 2^{-2} M sucrose and 2^{-2} M NaCl with quite similar impulse frequencies (table 4). This indicates that the response of the sugar-NaCl receptor to sucrose is not depressed in the presence of NaCl. T receptor discharged no impulses by ap cation of 2^{-2} N acetic acid, 1 mM quin hydrochloride in 10 mM NaCl, or 2^{-2} $MgCl_2$ (fig. 12). The slight response the mixture of 2 mM soluble sacchari and 5 mM NaCl (fig. 12C) might be d to the NaCl as in the sugar receptor. T spikes in response to NaCl or the mixtu of sucrose and NaCl seemed to be small in most cases in this hair (fig. 12B, D, I, J). Judging from homogeneity of the spikes in single records including the ca of mixed solution and comparable impu frequencies among the records, the spikes are regarded as originating in t same neuron, the sugar-NaCl receptor.

The response of the L neuron to sal acids, and alcohols has been reported the blowfly (Hodgson and Roeder, '56 The present sugar-NaCl receptor is som what different in its nature, in that a s gle chemoreceptor responds to both suga and non-sugars (cf. Dethier, '56).

We failed to identify any other chem receptor neurons which could be cor

TABLE 3

Impulse frequencies of a sugar-NaCl recepto sucrose and NaCl (from fig. 11)

Stimulant	Impulse frequency			
	Time after stimulus applied (s			
	2	3	4	5
2^{-2} M sucrose	30	25	25	20
2^{-4} M NaCl	33	28	24	17
2^{-2} M NaCl	34	26	20	15
2^{-6} M NaCl	17	10	8	8
1 M NaCl	29	21	15	14
2^{-2} M sucrose	23	19	16	15

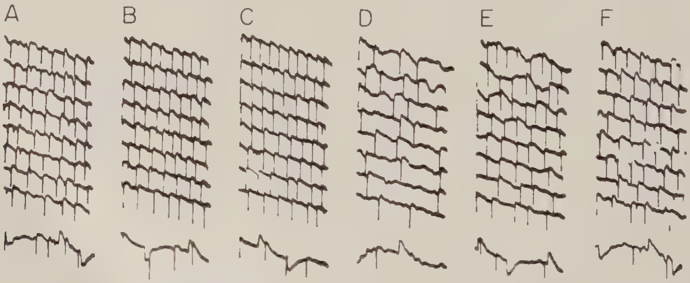

Fig. 11 Responses of a sugar-NaCl receptor to sucrose and NaCl. Responses: A and F, to 2^{-2} M sucrose; B, to 2^{-4} M NaCl; C, to 2^{-2} M NaCl; D, to 2^{-6} M NaCl; E, to 1 M NaCl. (Intervals ca. 5 minutes.)

TABLE 4

Impulse frequencies of a sugar-NaCl receptor to sucrose, NaCl, sucrose-NaCl mixture and qualities (from fig. 12)

Stimulant	Impulse frequency				
	Time after stimulus applied (sec.)				
	2	3	4	5	6
2^{-2} M sucrose	21	19	17	16	14
2^{-3} M NaCl	17	13	9	8	8
2 mM soluble saccharine·5 mM NaCl	5	3	3	2	2
1 M NaCl	14	9	8	6	6
2^{-2} N acetic acid	0	0	0	0	0
1 mM quinine hydrochloride·10 mM NaCl	0	0	0	0	0
2^{-2} M sucrose	21	19	19	17	16
2^{-2} M NaCl	21	17	14	12	9
2^{-2} M sucrose·2^{-2} M NaCl	22	21	19	18	18
2^{-2} M sucrose·2^{-2} M NaCl	25	21	21	20	18
2^{-2} M sucrose	24	25	22	20	19
2^{-2} M $MgCl_2$	0	0	0	0	0
2^{-2} M sucrose·2^{-2} M NaCl	25	21	18	14	12
2^{-2} M sucrose·2^{-2} M NaCl	23	20	17	15	11

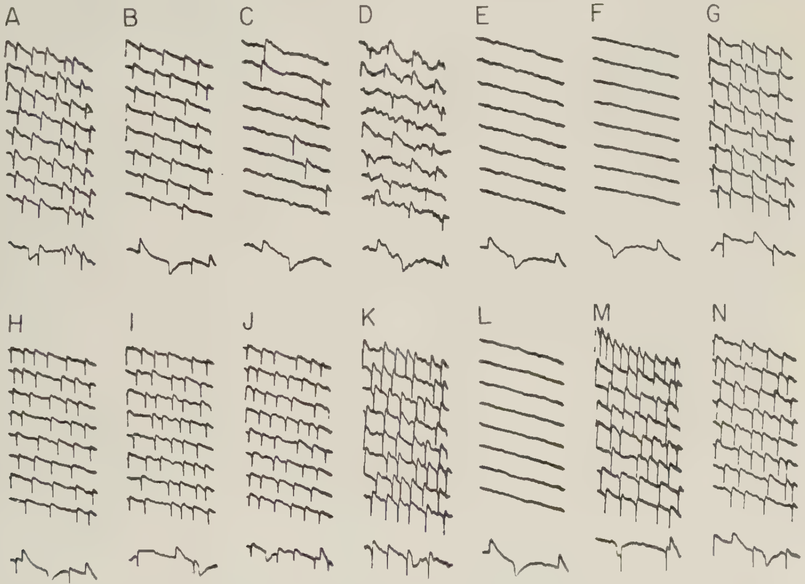

Fig. 12 Responses of a sugar-NaCl receptor to sucrose, NaCl, sucrose-NaCl mixture and qualities. Responses: A, G and K, to 2^{-2} M sucrose; B, to 2^{-3} M NaCl; C, to 2 mM soluble saccharine in 5 mM NaCl; D, to 1 M NaCl; E, to 2^{-2} N acetic acid; F, to 1 mM quinine hydrochloride in 10 mM NaCl; H, to 2^{-2} M NaCl; I, J, M and N, to 2^{-2} M sucrose in 2^{-2} M NaCl; L, to 2^{-2} M $MgCl_2$. (Intervals ca. 8 minutes.)

ated with the chemical stimuli used. Iowever, it may need further examina-ion to determine what other types of hemoreceptors exist.

In vertebrates single taste fibers comnonly respond to two or more qualities f taste stimuli (Pfaffmann, '41, '55; eidler, '53; Fishman, '57; Sato and Kuano, '60). Since, however, several taste receptors seem to be innervated by a single taste fiber, it is uncertain whether single taste receptors respond to different stimuli or not. Thus the taste reception in sugar receptor in *Vanessa* seems to be simpler than that in vertebrates. On the other hand, there is also a multiple sensitivity of a single receptor as in the sugar-NaCl receptor.

Behavioral tests in *Vanessa* showed that in some animals the response to sucrose was depressed in the presence of NaCl, and in other animals NaCl gave a positive response. Also the responsiveness in the same animals altered according to physiological conditions (Kuwabara, '52). In an animal which responded positively to NaCl, the presence of NaCl enhanced the sucrose response (Kuwabara, '51).

The depression by NaCl in behavior might simply be explained by the depression of activity in the sugar receptor. However, to explain the enhancing effect of NaCl, another mechanism which might be played by sugar-NaCl receptor should be taken into consideration. Thus, in this case, sugar-NaCl receptors may send excitatory impulses.

SUMMARY

1. Two kinds of spikes, the large and the small, are discriminated in single tarsal chemosensory hairs of the butterfly, *Vanessa indica*. The large spikes are from a chemosensory neuron and the small spikes from mechanoreceptor.

2. In most ventral hairs the large spikes are from a sugar receptor which responds specifically to sugars with fairly high and quite regular impulse frequency. The adaptation to sucrose is slower than to D-fructose.

3. Response to sucrose of the sugar receptor is depressed in the presence of NaCl of fairly low concentration, and is almost completely abolished by 1 M NaCl. This suggests a direct inhibitory action of NaCl on the sugar receptor.

4. The sugar receptor responds most strongly to sucrose, less to D-fructose and much less to D-glucose, but does not to trehalose, L-sorbose, inositol, D-galactose or lactose. It responds with deformed smaller spikes occasionally after a silent period to turanose or D-maltose, which suggests that these disaccharides may have both stimulating and inhibitory effects.

5. It is shown that D-glucose or D-fructose unit, especially the latter, is effective in stimulating the sugar receptor. Also, it is suggested that sucrose has some structural validity besides a simple sum of its monosaccharide constituents in sugar stimulation.

6. In some ventral hairs a sugar-N
receptor which responds with the la
spikes to sucrose and NaCl as well,
with slower adaptation to sucrose, is fou
instead of sugar receptor, and the respon
of which to sucrose is not depressed in
presence of NaCl.

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discussions.

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elationship Between Renal Succinoxidase Activity nd Maximal Transport Rates of p-Aminohippurate Tm_{PAH}) in Various Representative Vertebrates[1]

ROY P. FORSTER AND LEON GOLDSTEIN[2]
Department of Zoology, Dartmouth College, Hanover, New Hampshire and the Mount Desert Island Biological Laboratory, Salisbury Cove, Maine

Interest in this problem arose from the
rtuitous observation that kidneys of ma-
ne fishes maintained under laboratory
nditions continue to excrete at essen-
ally undiminished rates such actively
ansported organic acids as p-aminohip-
uric (PAH) and the phenolsulphonphtha-
ins (Forster, '53) despite the progres-
ve drop in hematocrit to less than 5%
hich characteristically accompanies the
ttending diuresis and shift in the electro-
tic composition of their body fluids dur-
g captivity (Forster and Berglund, '56).
ince this transport process has been
nown to be dependent upon tricarboxylic
cid cycle-cytochrome system activity and
erobic phosphorylation (Forster and Tag-
art, '50; Taggart and Forster, '50), the
aintenance of maximal secretory rates
ight be due to an adaptive increase in
ne or more enzymes of the T.C.A. cycle.
owever, it was found that the activity
f a representative T.C.A. cycle enzyme
ystem, succinoxidase, was not signifi-
antly altered by reduced blood oxygen
ontent. Comparative studies revealed a
ignificant correlation between maximal
enal succinoxidase activity and the max-
mal capacity of renal tubules of various
pecies to transport PAH.

METHODS

Techniques as described earlier were
sed for the excretion studies in *Lophius*
Forster, '53). The goosefish were ob-
ained by dragging an otter trawl at
epths of about 200 feet. After capture
hey were maintained in laboratory
quaria supplied with rapidly running
old sea water. All injections and the
ollections of blood and urine were made
without removing the fish from water. Urine was obtained by indwelling catheters which were plugged during collection periods, allowing urine to accumulate in the bladder, and unplugged at the termination of the experimental periods. Either arterial or venous blood was obtained ventrally with a 25-gauge needle from caudal vessels of the tail. The absence of glomerular filtration in this fish permitted calculation of Tm_{PAH} simply as the total amount of PAH excreted, which was expressed in μmoles/kg/min.

Isolated renal tubules of the flounder were used for the observations on malonate inhibition of phenol red transport as described by Forster and Taggart, ('50). The homogenate technique for measurement of succinoxidase activity in the various cortical renal tissues was as originally described by Schneider and Potter, and as published in Umbreit et al. ('57). Blood oxygen content was measured by a micromanometric technique described by Holaday and Verosky ('57). Deoxyribonucleic acid was measured as previously described by Goldstein et al. ('56).

RESULTS

As shown in figure 1, there was no relationship between blood oxygen concentrations, which ranged between 1.5 and 7 vol. per cent in blood supplying the Lophius kidney, and Tm_{PAH} or renal succinoxidase activity. In this experimental series 8 goosefish were examined for Tm_{PAH} one at a time, starting with the first a few hours after capture, and then continuing with one each day over a period of a week while hematocrits and blood oxygen values fell during the course of spontaneous "laboratory diuresis." Blood collections were taken and renal succinoxidase activity measured immediately following each urine collection period. Renal succinoxidase activity was expressed per mg DNA to correct for changes in cellularity that might have occurred during the experimental period (Tappan et al., '57). Data is included in figure 1 from 7 animals in another series which were handled similarly, one being sacrificed each day, but without Tm_{PAH} measurements being made. The fact that undiminished rates of PAH secretion were maintained in kidneys supplied with venous blood containing as little as 1.5 vol. per cent oxygen suggested that an adaptive response might have occurred in the T.C.A. cycle enzyme system, succinoxidase, as was previously observed in guinea pigs acclimatized to low oxygen tensions (Tappan et al., '57), and that this might have furnished sufficient energy to maintain normal Tm_{PAH} levels. However, when the assays of succinoxidase activity indicated no significant alteration expressed either on a DNA or fresh wei basis throughout this wide range of bl oxygen levels, an alternate hypothesis entertained; that even at these very values oxygen content was not a limit factor in the maintenance of PAH tra port rates. Actually, the ratio of Tm_{PA} blood oxygen concentration at the low values per cent of oxygen in Lophius similar to that in normal mammals. the dog, for example, with about 10 × oxygen content in arterial blood the Tm of 5 μmoles per kg per minute is com rable with 0.5 μmoles per kg in Loph (Smith, '51).

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[2] Present address: Department of Biochemistry, Harvard Medical School, Cancer Research Institute, New England Deaconess Hospital, Boston 15, Massachusetts.

To test the hypothesis that rate lim tion of maximal PAH transport might due to total capacity of Krebs' cycle ac ity, Tm_{PAH} values for other representat vertebrates were gathered from the lite ture and then succinoxidase activity measured in homogenates of renal c cal tissue taken from these animals. S values for Tm_{PAH} expressed in μmoles kg per minute were obtained for the cat, dog (Smith, '51) and frog (Schm Nielsen and Forster, '54), and as seen figure 2, they show a linear relations when plotted against succinoxidase ac ity expressed in μl O_2 per 100 mg fr weight of renal cortical tissue per minu

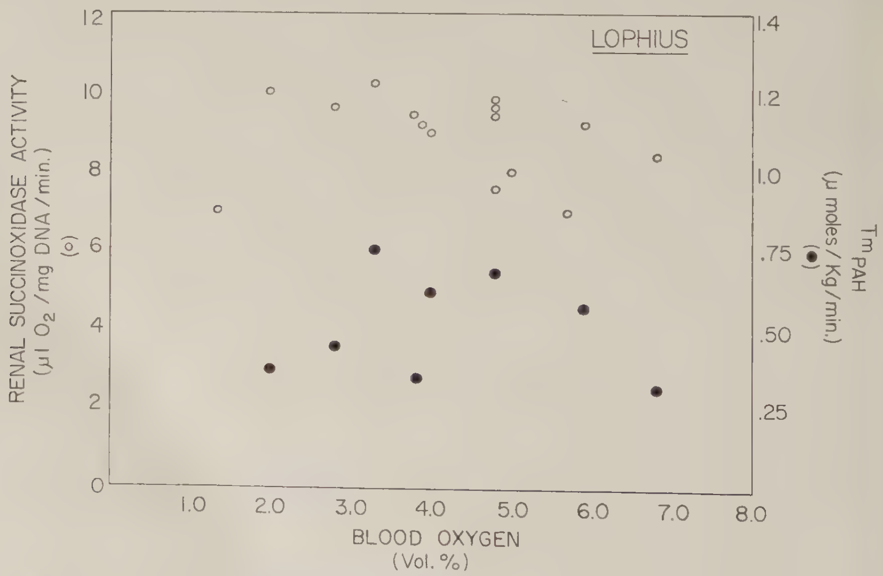

Fig. 1 Relation of renal succinoxidase activity and Tm_{PAH} to blood oxygen content.

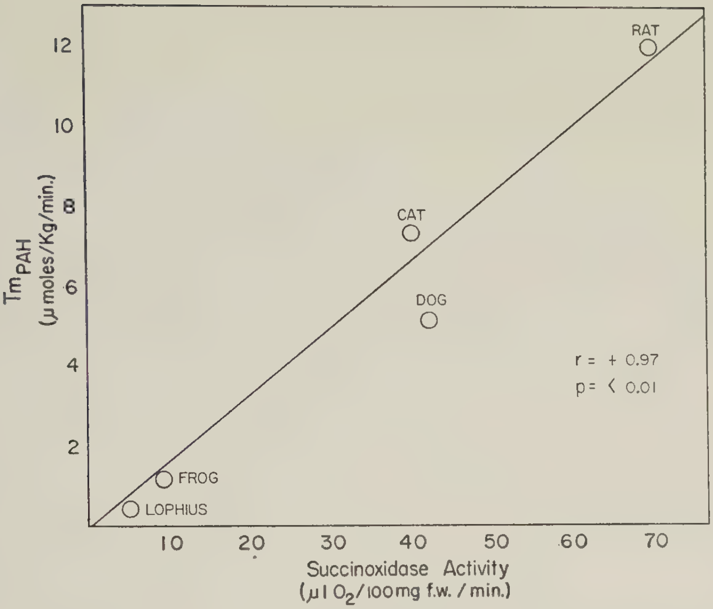

Fig. 2 Relationship of renal succinoxidase activity to Tm_{PAH} in various vertebrates.

Malonate which selectively inhibits the
idation of succinic acid was used in the
olated tubule preparation to test its effect
transport of phenol red, another or-
nic anion competitively transferred by
e hippurate system (Forster and Tag-
rt, '50). Within 30 minutes of exposure
e *in vitro* preparation was reversibly in-
bited by M/50 and M/100 solutions of
alonate, with the higher concentration
ing the more effective inhibitor. Upon
nsfer to control medium, total recovery
as achieved within 60–90 minutes after
e inhibition of luminal dye accumulation
had been definitely ascertained. M/50 glucose added to the balanced isotonic salt solution served also as a control to test for possible non-specific osmotic effects.

GENERAL DISCUSSION

Since it was first shown that various inhibitors of aerobic phosphorylation were also effective in blocking phenol red transport (Taggart and Forster, '50), many studies during the past decade have pointed to the tricarboxylic acid cycle-cytochrome system as the source of free energy required for the work done in the

TABLE 1

Reversible inhibition of phenol red transport by malonate in the flounder isolated tubule

Time	Control	M/50 Glucose	M/50 Malonate	M/100 Malonate	M/50 Malonate	M/100 Malonate
min.						
15	+	+	+	+	+	+
30	+++	+++	+	++	+	+
					Transferred to control medium	
90	++++	++++	+	+++	++++	+++
180	++++	++++	0	+	++++	++++

Semiquantitative evaluations of dye accumulation in lumen are indicated as ranging from 0, no concentration, to ++++, maximal concentration. Malonate was added to the control balanced isotonic medium, and glucose was used to test for possible nonspecific osmotic effect of the added solute.

organic anion transport process, and to ATP as the form in which energy is captured and harnessed by the cell. However, little has been learned since about how this energy is brought to bear on the transport mechanism itself. In all vertebrates the cell membrane on the vascular side of proximal tubule cells appears to be an energy demanding locus which is also the site of competitive inhibition, and in cold blooded vertebrates the luminal cell membrane additionally is the site of other specific "carriers" with turnover characteristics which are similar to, but not identical with, those on the vascular side of these cells (Hong and Forster, '58). An intracellular trapping mechanism also appears to constitute a separate phase which has been implicated especially with the movement of slowly transported anionic and cationic dyes in both the isolated tubules of the flounder (Forster and Hong, '58) and in thin slices of the rabbit renal cortex (Copenhaver and Forster, '58). Since transport across the membrane on the vascular side of proximal cells is normally the rate limiting step and the only active site shared by all the vertebrates which show a direct correlation between succinoxidase activity and Tm_{PAH}, and inasmuch as the inhibitory effects of malonate are also noted here, it is reasonable to suppose that aerobic phosphorylative activity is brought to bear on the transport mechanism at this locus. Mitochondria, which contain all the components needed to sustain succinoxidase activity and the attending electron flow to ATP synthesis, are shown by electron micrographs to be very intimately associated with the basal or vascular part of proximal cells. Actually this part of the cell is thrown into clefts by infoldings of the cell membrane which result in cytoplasmic lamellae resting on the basement membrane, with densely packed mitochondria in close association and tightly squeezed between them (Rhodin, '58). With essentially nothing known either of the chemical reactions an organic acid undergoes during transport, or of the nature of the carrier itself, it is useless at this time to speculate on how interaction is achieved between the energy
donating system and the transport
chine which does the electrochemical w
This clearly is one of the important f
points of interest for all cell biologists
cerned with active transport problems

Malonate, which specifically inhibits
oxidation of succinic acid, has previo
been shown by Shideman and Rene ('
to inhibit PAH and phenol red trans
in vivo in the dog, and *in vitro* in ra
and guinea pig slices. A close correla
was shown to exist between the concen
tion of inhibitor needed to depress suc
oxidase activity and the doses requi
to suppress transport of these orga
acids. Dominguez and Shideman ('
similarly noted that, after administrat
of the inhibitor to intact rats, *in vi*
preparations of cortex slices exhibite
linear relationship of transport depress
to doses which produced as much as 6
inhibition of the PAH slice to medium c
centration ratio. Considerable specifi
in the succinoxidase-Tm_{PAH} relations
was revealed by the inability of malon
to affect glucose or phosphate reabsorpt
in doses which simultaneously depres
the tubular secretion of organic anio
This difference in the energy requireme
of secretory and reabsorptive proces
was also suggested by the observation
Mudge and Taggart ('50) that adminis
tions of 2,4-dinitrophenol which marke
reduced the tubular secretion of PAH,
drast and phenol red in dogs had no eff
on the simultaneous reabsorption of
cose or glycine. Furthermore, specifi
with respect to dependence of secret
processes on energy sources was indica
by the finding that tubular secretion
potassium, measured in dogs during
infusion of potassium salts, was not
fected by 2,4-dinitrophenol (Mudge
Taggart, '50), and maximal secretory ra
of the organic base, tetraethyl-ammoni
ion, were unaffected by such Krebs cy
inhibitors as malonate and fluoroacet
(Renick and Farah, '56). These obser
tions suggest that specific and intim
relationships exist between cell meta
lism and the specialized functions wh
characterize renal function, but the nat
of these associations remains obscure.

SUMMARY

omparative data from *Lophius*, frog,
, cat and rat show a significant correla-
(P < 0.01) between Tm$_{PAH}$ and renal
cinoxidase activity, with the goosefish
ing the lowest Tm$_{PAH}$ (0.5 μM/kg/min.)
the lowest renal succinoxidase activity
μl O$_2$/100 mg f.w./min.). Comparable
ues for transport and Krebs cycle activ-
in the dog are 10 times greater than in
, and in the rat these rates are more
n twice those of the dog.

n *Lophius* no decrease in Tm$_{PAH}$ nor in
al succinoxidase activity accompanied
s in blood oxygen content from 5 vol.
cent down to 1.5, when during 4–6
periods of capitivity hematocrits
pped as low as 5%.

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Quantitative Study of the Distribution Pattern of rtain Oxidizing Enzymes and a Lipase in the d and White Fibers of the Pigeon east Muscle

J. C. GEORGE AND C. L. TALESARA
Division of Animal Physiology and Histochemistry, Department of Zoology, M. S. University of Baroda, Baroda, India

hat certain structural and biochemical erences exist in the red and white mus- of vertebrates in general, is known n the works of a number of investiga- (Needham, '26; Denny-Brown, '29; l and Sperling, '52; Lawrie, '52; Ed- d et al., '56; Nachmias and Padykula, Tonzetich and Kare, '60; Ogata, '60 and oowitz and Pearse, '60). These differ- es have been studied by employing his- emical or biochemical methods mostly the mixed types of muscles or the red white muscle portions taken sepa- ly either from different muscles of the e animal or from different animals not on the fibers isolated from a mixed scle. It was observed that in every such ce of muscle its cellular components not differ in their diameter, metabolite e and various other constituents but differ markedly in the ratio of their ribution pattern in the different re- ns of the same muscle (George and k, '57a, '59; George and Talesara '60; Talesara, '61). It was suggested there- that the results cannot be obtained stant in a muscle of this type or any er muscle unless the ratio of the distri- ion pattern of the different types of rs is taken into consideration.

Recently Ogata ('60) showed the differ- es in some labile constituents and en- atic activities between the red muscle en from the central part of M. *soleus* the white muscle taken from the pos- or edge of M. *adductor magnus*. The ults obtained by the author do throw e light on the general nature and be- ior of the two types of muscles taken arately, but in a mixed muscle like the pigeon pectoralis it is worthwhile investigating quantitatively by some suitable methods the various constituents in the individual fiber types since the isolation of these fibers without damaging them appears extremely difficult. In a histochemical study of the various DPN-linked dehydrogenases (George and Talesara, '61a) and succinic dehydrogenase and cytochrome oxidase (George and Talesara, '61b), employing more sensitive and modified methods, it was possible to demonstrate the localization of these enzymes in the broad white fibers of the pigeon breast muscle, previous attempts to accomplish this (George and Scaria, '58) having failed.

To put these observations to further test the present investigation on the activities of the various enzymes such as cytochrome oxidase, succinoxidase, malic oxidase, lactic dehydrogenase and lipase has been carried out in the different layers of the pigeon breast muscle employing the indirect method (George and Talesara, '60; Talesara, '61) of assessment without isolating the fibers.

MATERIAL AND METHODS

In order to ensure that only uniformly well developed *pectoralis major* muscle was used, well fed and fully grown, normal laboratory pigeons (*Columba livia*) of either sex weighing between 300 to 350 gm were used.

In each experiment a bird was decapitated and the blood completely drained off. A muscle strip of nearly 2 cm^2 cut along the direction of the fibers was removed from the middle of the *pectoralis major* muscle with its entire thickness.

Similarly another piece with exactly the same topography was quickly cut from the muscle of the other side. One of these was used for the assay and the other for recording the distribution of the broad and narrow fibers in the different layers. Slices of desired thickness were cut immediately on a freezing microtome and the fiber count of the narrow as well as the broad fibers was taken according to the procedure cited earlier (George and Talesara, '60). A 1 to 2% homogenate out of each muscle slice representing a particular depth was immediately prepared in ice-cold glass redistilled water. The same steps in the procedure were followed for all the enzymes studied. In the case of lipase special care was taken to remove the traces of blood completely.

Oxidizing enzymes

Oxidizing enzymes such as cytochrome oxidase, succinoxidase, malic oxidase and lactic dehydrogenase were assayed at 37°C with the conventional Warburg manometric techniques through oxygen uptake. In each case the Warburg flask contained 3 ml of the total reaction mixture in the main chamber and 0.2 ml 6 N KOH and a roll of filter paper in the center well. The gas phase used was air and after temperature equilibration in the bath for 10 minutes, the levels were adjusted and the oxygen uptake was recorded at 10-minute intervals for 30 minutes.

The enzyme activity is expressed in microliters of oxygen consumed per milligram dry muscle per hour. The dry weight of the homogenate was determined on 1.0 ml duplicate samples dried to constant weight at approximately 100°C. Glass redistilled water was used for preparing all the reagents used. Two-tenths to 0.4 ml of approximately 1% homogenate was used in each case for the enzyme assay. Endogenous oxygen uptake for this small amount of tissue used was found to be almost negligible. All the chemicals used in the assay were of routine C. P. grade (Merck) except DPN and cytochrome *c* which were of the Sigma Chemical Co.

Cytochrome oxidase

The activity of this enzyme was measured by the manometric method of Schneider and Potter ('43). The principle
which the assay depends is that a
enzymatic reduction of cytochrome
ascorbic acid is used to serve as the
strate for cytochrome oxidase. The
rection for the oxygen uptake resul
from the spontaneous auto-oxidation
ascorbate was made as suggested by
authors.

Succinoxidase system

Succinic dehydrogenase, the Slater
tor and the cytochrome oxidase sys
constitute the succinoxidase system.
manometric technique described by Sch
der and Potter ('43) was used for de
mining the enzyme activity.

Malic oxidase system

Malic dehydrogenase and the associ
hydrogen carriers, DPN, cytochrome
ductase and the cytochrome oxidase
tem constitute the malic oxidase sys
The overall activity of this complex of
zymes was determined according to
manometric technique of Potter ('
Glutamate was added to remove oxal
tate by means of the transamination
action (Straub, '41) and nicotinamide
added to inhibit DPN destruction (M
and Quastel, '41).

Lactic dehydrogenase

The enzyme activity was determ
manometrically through oxygen uptak
the method adopted from the report
Straub ('40), Vestling and Augusta (
and Hunter and Hunter ('57). The r
tion flask contained:

	ml
0.1 M PO_4 buffer, pH 7.6	0.8
3.5×10^{-4} M cytochrome *c*	0.5
0.5 M DL-lactate	0.8
1% DPN	0.3
Muscle homogenate	0.4

Glutamate was also included in s
experiments for removing pyruvic acid
no significant increase in the oxygen
take was observed.

Lipase activity

Lipase activity of the homogenate
determined manometrically by the me
adopted from Martin and Peers ('53)
a bicarbonate-carbondioxide buffer sys
of pH 7.4 at 37°C using the Warburg

atus. The reaction flask contained 1.5 0.025 M bicarbonate solution and 1.0 homogenate in the main chamber and ml 4% (v/v) tributyrin in 0.0148 M arbonate solution (emulsified by shak- with a drop of "Tween" 80) in the side n. The flasks and the manometers were ssed with a mixture of 5% carbon di- de and 95% nitrogen for three minutes. e substrate was tipped in after equilibra- n for 10 minutes and after equilibration another three minutes the levels were usted and the readings taken at regular ervals of 15 minutes for one hour. The ase activity is expressed as µl CO_2/mg muscle/hour.

RESULTS

The activities of the various enzymes died and the number of broad and rrow fibers per square millimeter in the ferent layers of the pigeon breast muscle shown in table 1. The results so ob- ned clearly indicate that with the vari- on in the ratio of the distribution of the oad and narrow fibers, the concentra- ns of the various enzymes studied also ry accordingly. The activity of these zymes was found to be minimum in the 5 mm thickness of the most superficial ers where the broad fibers are maxi- um, whereas it was maximum in the er taken from the interior of the mus- . Thus a definite pattern in the distri- tion of the two types of fibers and their sociated constituents is observed in this uscle.

Figures 1–3 are a graphic representa- on of the ratio of narrow and broad fibers ainst the activity of the various enzymes obtained from different layers of the uscle. George and Naik ('59) showed at per unit area of cross-section of the uscle at any particular depth, the num- r of broad fibers is inversely proportional the number of narrow fibers and de- ved the formula

$$Y = -5.75\,X + 890.01$$

vhere Y stands for the number of nar- w fibers and X for the number of broad bers). It means that when X is zero the tal number of narrow fibers per square illimeter of area would be 890, i.e., all arrow fibers and when Y is zero, the total

TABLE 1

Showing the distribution pattern of the various enzymes in the different layers of the pectoralis major muscle of the pigeon and its relation to the number of muscle fibers (The figures indicate the average values of 5 sets of experiments in each case)

Exp.	Depths of the muscle starting from the superficial side (ventral face)	No. of fibers per mm²		Oxidizing enzymes: µl O_2 uptake/mg dry muscle/hour				Lipase activity µl CO_2/mg dry muscle/hour
		Broad	Narrow	Cytochrome oxidase	Succin-oxidase	Malic oxidase	Lactic dehy-drogenase	
	mm							
1	0–0.5	119 ± 11	214	146.59	37.90	35.98	20.44	19.08
	0.5–1.0	88 ± 9	385	238.24	60.76	57.52	30.34	29.31
2	0–1	102 ± 8	301	198.86	48.74	45.34	25.90	24.64
	1–2	78 ± 6	471	295.28	68.40	70.77	35.52	35.40
	2–3	56 ± 7	567	340.54	86.03	81.76	42.07	41.46
	3–4	44 ± 3	640	387.95	93.92	91.70	46.18	46.54
	4–5	34 ± 6	696	416.15	104.55	98.73	49.24	51.18

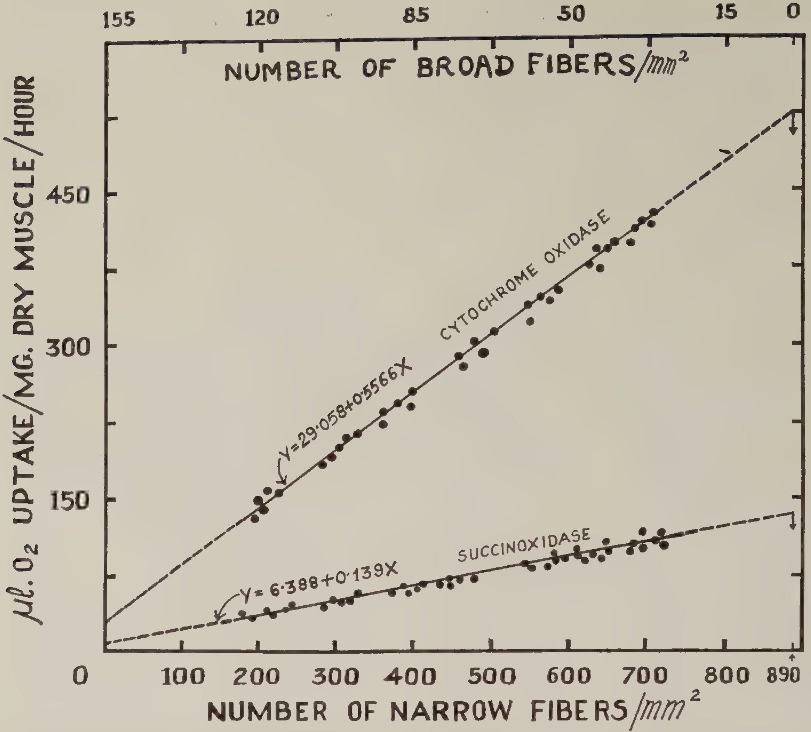

Fig. 1 Graph showing the relation between the number of narrow and broad fibers per square millimeter and cytochrome oxidase and succinoxidase activity as obtained at different depths of the *pectoralis major* muscle of the pigeon.

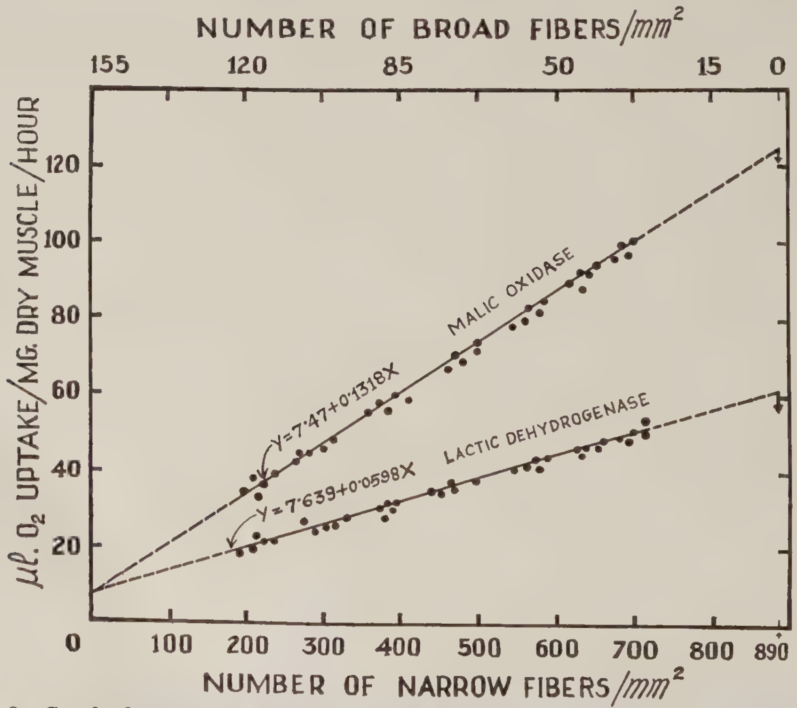

Fig. 2 Graph showing the relation between the number of narrow and broad fibers per square millimeter and malic oxidase and lactic dehydrogenase activity as obtained at different depths of the *pectoralis major* muscle of the pigeon.

mber of broad fibers per square milli- ter of area nearly 155, i.e., all broad ers. It should be mentioned, however, at this is merely a theoretical assump- n and such a condition never exists in s muscle in any region. The use of the gression line so obtained in each case, s made for deriving the enzyme activi- s in the individual red and white fibers, extending the regression lines on either le as indicated by broken lines (figs. 3). The activities of the various en- mes so derived in the individual red and ite fibers are shown in table 2. It should, however, be noted that the values derived by this indirect application of the method may not be absolute since the freezing and thawing of the muscle piece while cutting, though for a very short period, might destroy some amount of the enzyme. Nevertheless, the results obtained do show the relative enzyme concentrations in the two types of fibers of this muscle.

From the results obtained (figs. 1–3 and tables 1–2) it could be easily noticed that the major bulk of all the enzymes studied is confined to the narrow red fibers of this muscle though the broad white fibers too

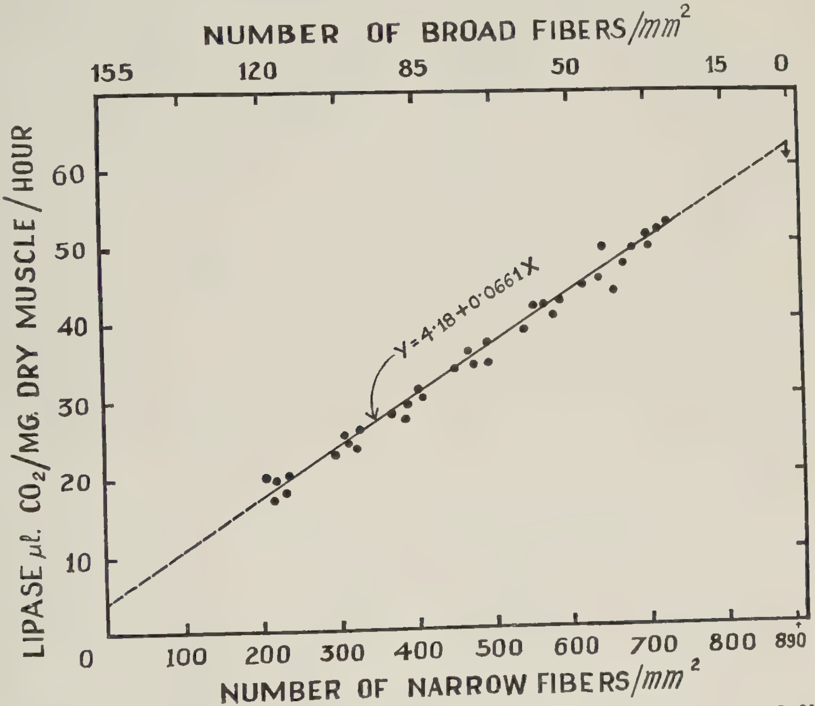

Fig. 3 Graph showing the relation between the number of narrow and broad fibers per square millimeter and lipase activity as obtained at different depths of the *pectoralis major* muscle of the pigeon.

TABLE 2

Showing the activity of the various enzymes in the red as well as the white fibers of the pectoralis major muscle of the pigeon as derived from the equation of the regression line

Muscle	Oxidizing enzymes: μl O_2 uptake/mg dry muscle/hour				Lipase activity: μl CO_2/mg dry muscle/hour
	Cytochrome oxidase	Succinoxidase	Malic oxidase	Lactic dehydrogenase	
Red	524.43	130.10	124.77	60.87	63.00
White	29.05	6.38	7.47	7.63	4.18

possess all these enzymes, but in extremely low concentrations as was shown by histochemical methods also (George and Talesara, '61a and '61b).

DISCUSSION

A higher concentration of the oxidizing enzymes and a lipase in the narrow red fibers, suggests the presence of a well organized cyclophorase system including specially the enzymes of the Krebs cycle and fat utilizing system whereas the broad white fibers are poor in these contents. George and Naik ('58a and '58b) have shown that the broad white fibers are glycogen-loaded and poor in fat inclusions and mitochondria whereas the narrow red fibers are fat-loaded and have a high mitochondrial content and are poor in glycogen. Based on these observations it could be said with fair amount of certainty that the oxidation of fatty acids in the pigeon breast muscle takes place mostly if not entirely in the narrow red fibers. With a high fat content and its associated enzymes it is more or less certain that fat forms the chief fuel for energy in preference to glycogen during long and sustained contractions of the narrow red fibers of the pigeon breast muscle and the same appears to be the case in the flight muscles of birds indulging in such prolonged muscular activity as in migratory flights. The activities of the various enzymes and the amounts of the constituents assessed might appear to be very low in the broad white fibers as compared to the narrow red fibers, but these values for the broad white fibers are quite comparable to those obtained for the white fibers of other vertebrate skeletal muscles.

In a mixed muscle like the pigeon pectoralis and other vertebrate skeletal muscles in general, the distribution in a definite fashion of the fiber types with their associated constituents within, cannot be considered as without any significance. In the pigeon breast muscle as has been observed, the broad white fibers are concentrated more towards the periphery in a single fasciculus and this number increases to the maximum in the fasciculi situated in the superficial and the deepest layers. Thus the number of the bro fibers is the least in the fasciculi situat in the interior of the muscle. This is su cient evidence to demonstrate by expe mental data that variations in the diffe ent metabolites, enzymes and other cellul constituents do exist and are considerab higher in the different regions of th muscle. It should be noted, however, th this variation is not random but follow a definite distribution pattern (table and not a unique case in the *pectora major* muscle of the pigeon but may occ in other muscles too as is reported in t *pectoralis major* muscle of the bat (Geor and Naik, '57b).

The results obtained for the differe layers of the pigeon breast muscle indica that the metabolically most active cente lie somewhere in the middle of the dors ventral axis where the number of the na row fibers is the highest and that of th broad fibers the least. Hence the gener belief that the muscle fibers towards th periphery of the muscle are more acti than those in the interior and, due higher activity increase in diameter ca not be accepted as a universal rule ar at least in the case of the *pectoralis maj* muscle of the pigeon this does not ho good. The broad white fibers which a more concentrated towards the periphe of the muscle and have more of the stru tural protein (Talesara, '61) as compare to the narrow red fibers, are mechanical more efficient in quick contractions for short duration. Hence the variation in th fiber types accompanied by variations their metabolite load in a definite patte may well be associated with the mecha ical and physiological efficiency of the d ferent fibers and ultimately of the musc as a whole.

CONCLUSIONS

1. The distribution pattern of the tw types of fibers as well as the activities the various enzymes such as cytochrom oxidase, succinoxidase, malic oxidase, la tic dehydrogenase and lipase were studi in the different layers of the pigeon brea muscle.

2. All the enzymes studied were foun to vary in their concentrations in the di ferent layers depending on the variatio

the ratio of the distribution of the two pes of fibers.

3. The results indicate that the meta- olically most active centers lie in the terior of the muscle where the narrow bers are the highest in number and the road fibers the least and that the fibers owards the periphery of the muscle show poor capacity for respiratory metabolism.

4. From the statistical analysis of the esults obtained for the different layers, he activities of the various enzymes are erived for the fiber types with the help f the regression line, without actually aving to isolate the fibers.

5. The values derived for the individual ed as well as white fibers indicate that he major bulk of all the enzymes studied esides in the red fibers whereas the white ibers too possess these enzymes but in ery low concentrations.

6. The distribution pattern of the two ypes of fibers and their associated inclu- ions in a definite fashion indicate the echanical and physiological efficiency of his muscle.

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essation of Renal Function During Diving in the rained Seal (*Phoca vitulina*)[1]

H. V. MURDAUGH, Jr., BODIL SCHMIDT-NIELSEN,[2] J. W. WOOD and
W. L. MITCHELL

*Mt. Desert Island Biological Laboratory, Salisbury Cove, Maine and
Department of Medicine, University of Alabama Medical Center and
Department of Zoology, Duke University*

The adaptive response of the seal to
ving appears composed of two changes,
reflex bradycardia with resultant de-
ease in cardiac output reported by Scho-
hder ('40), and an arterial constrictor
sponse with cessation of blood flow
rough muscle beds described by Irving,
holander, and Grinnell ('41). These
anges allow the seal to maintain a
gher oxygen saturation of blood perfus-
g the brain for a longer period of time.
holander ('40) thought this response
as of a reflex nature because brady-
rdia may appear within one heart beat
submersion.

Bradycardia of diving has been experi-
entally produced by different techniques.
holander ('40) studied trained seals
ved on a teeter board, but Lowrence,
ickel, Smythe and Bradley ('56) ob-
ructed respiration by placing an oc-
uded cone over the seal's nose to obtain
adycardia. The electrocardiograms dur-
g these two conditions differ. The ac-
stomed dive of the trained seal on the
eter board resulted in a bradycardia
ithout arrythmias (Scholander, '40), but
spiratory obstruction resulted in brady-
rdia of lesser degree but accompanied
T wave inversion and arrythmias (Low-
nce, Nickel, Smythe and Bradley, '56).
lectrocardiograms taken on the free
vimming seal (Murdaugh, Seabury and
itchell, '61) were like the electrocardio-
rams taken by Scholander using teeter
oard diving.

Bradley and Bing ('42), using the oc-
uded nose cone technique and forced
ubmersion in the untrained seal reported
ecreases in rates of urine flow and glo-
erular filtration in the seal. In our previous study (Schmidt-Nielsen, Murdaugh, O'Dell and Bacsanyi, '59) attempts were made to determine the effect of diving on renal function using the occluded nose cone technique, but the violent struggling of the seal made interpretation of the data difficult. It was noted that with struggling there was a decrease in urea to creatinine clearance ratio similar to the findings in the white rat when frightened.

The present study was designed to determine the changes, if any, in renal function during diving in the trained seal. It was found that when the trained seal was dived on a teeter board urine flow stopped. The evidence obtained suggests cessation of renal blood flow during diving.

METHODS

Five young female harbor seals were studied. They were kept in a live car in the bay, fed fresh herring and given penicillin to prevent subcutaneous infections that may develop in captivity. The seals were trained to dive on a teeter board using a method similar to that described by Scholander ('40). An aquarium was constructed with plastic sides to allow observation during submersion. The seals were laced comfortably to the teeter board in a manner that allowed the head and front part of the body to be submerged, leaving the posterior third of the seal out of water. They were each submerged for progressively increased periods of time until they would remain under water 10 minutes without struggle.[3]

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[1] Supported by a grant from the U.S.P.H.S.

[2] Established investigator of American Heart Association.

[3] It was of interest to the investigators that this training period required only two weeks.

A priming dose of creatinine and p-aminohippurate (PAH) and maintenance infusions were administered via an inlying intravenous Rochester needle in a fore-flipper. The maintenance infusions were 5% mannitol given by constant infusion pump at a rate of 4 ml/min. with adequate creatinine and PAH to maintain plasma concentrations of approximately 20 mg/100 ml creatinine and 2 mg/100 ml PAH.

Blood was obtained by venipuncture from the extradural vein through an area of skin anesthetized with meticaine (R) which does not cause colorimetric interference with PAH determinations. This method of venipuncture was described by Harrison ('57) and was found to be the best way to obtain seal blood during diving.

Urine was collected with a Foley catheter using air washouts. A Foley catheter may make urine collections difficult in the seal if the balloon is inflated to a full 5 ml because the balloon will cover the ureteral orifices. Then one cannot empty the bladder completely and urine might leak around the catheter when the bladder is thought to be empty. It was found that no difficulty was encountered if the balloon was inflated to only 2½ to 3 ml. The bladder emptied well with the seal on the teeter board in the head down position.

Electrocardiograms were recorded using subcutaneous needle electrodes as described in a previous report (Murdaugh, Seabury and Mitchell, '61). Creatinine was measured using the method of Bosnes and Taussky ('45). PAH determinations were performed by a modification of the method of Bratton and Marshall ('39). Sodium analyses were made with a flame photometer.

RESULTS

Ten diving studies were performed and all gave similar results. The data from three experiments are depicted in table 1 representing examples of data obtained: when long collection periods followed the dive, when short collection periods followed the dive, and when atropine was administered.

It was found that urine flow stopped within one minute of the time the head was submerged. No urine was formed during the remainder of 10 minute divin periods but urine flow returned within tw minutes of end of submersion.

If the maintenance infusion of crea nine and PAH continued during diving th plasma concentrations of creatinine an PAH increased. Stable blood levels coul be obtained during the experiments on if the maintenance infusion was disco tinued during diving.

Creatinine and PAH clearances appear normal following the dive when long urin collections were obtained. When sho urine collections of two to 4 minutes we obtained following a dive, it was foun that both clearances were considerably r duced, but showed a stepwise return normal in about 20 minutes.

In the post-dive periods there was a d crease in the creatinine U/P and a bri diuresis and natriuresis without increa in urine sodium concentration.

When diving was performed 30 minut after intramuscular atropine (0.08 mg/kg bradycardia did not occur and urine flo continued without decrease in creatinin or PAH clearances during the dive. The findings were not in agreement with th report of Bradley and Bing ('42) of ren clearances during respiratory obstructio

DISCUSSION

The results show that diving causes a immediate and complete cessation of urin production in the seal. This is seen fro the fact that no urine could be collecte from the first minute after the head wa submerged and throughout the remaind of the diving period.

Whenever urine is not obtainable on must consider collection difficulties. Ho ever, the findings that urine was obtaine with ease during head down tilt withou water in the aquarium, and that clearanc returned toward normal in a step-wi fashion after diving indicate that the fa ure to obtain urine was not caused by co lection errors.

The absence of urine production a pears to be caused by a cessation of gl merular filtration and not by an increas in tubular reabsorption of water. This apparent from the findings that the creat nine clearance as well as the creatinin U/P ratios in urine obtained immediate

TABLE 1

Time	Urine flow	Exogenous creatinine		PAH		Urine sodium	
		U/P	C_{cr}	Urine conc.	C_{PAH}	mEq/l	Eq/min.
min.	*ml/min.*			*mg %*			
			Seal L 2, 7/13/60				
0–20[1]	2.95	45.8	135	107	316	191	0.56
20–29	3.61	42.0	152	91	338	207	0.75
29–39	3.65	37.9	138	79	343	190	0.70
S 46–52	0.25	36.8	9	85	21	180	0.04
S 52–56	0	—	0	—	0	—	—
56–66	5.25	17.7	89	47	247	139	0.73
66–81	4.67	23.9	111	51	238	172	0.80
S 83–92	0	—	0	—	0	—	—
92–103	4.00	16.4	66	36	142	162	0.65
103–115	4.67	23.2	108	—	—	180	0.84
			Seal L 2, 7/19/60				
0–14[1]	1.68	60.6	102	138	422	225	0.38
14–27	2.42	69.0	167	140	557	220	0.53
27–42	2.40	69.0	165	133	480	211	0.51
S 46–54	0	—	0	—	0	—	—
54–67	6.16	26.8	165	75	350	175	1.08
67–80	3.84	39.1	150	91	407	207	0.79
80–94	2.14	56.8	122	117	367	243	0.52
S101–111	0	—	0	—	0	—	—
111–115	3.25	26.4	86	71	170	224	0.73
115–118	3.33	19.1	64	54	134	190	0.63
118–124	3.33	22.6	75	70	184	175	0.58
124–139	3.46	28.6	99	91	279	204	0.70
139–150	2.46	45.7	112	107	273	217	0.53
			Seal R 2, 8/12/60				
0–11[1]	1.92	83.6	146	210	368	172	0.33
11–21	1.71	85.6	145	226	420	149	0.26
21–32	2.46	64.0	157	189	423	117	0.29
43–55	3.72	42.3	157	112	398	122	0.45
S 57–64	0	—	0	—	0	—	—
S 64–66	0	—	0	—	0	—	—
66–68	0.75	35.3	26	80	41	113	0.08
68–75	1.43	24.4	35	86	95	92	0.13
75–81	2.30	24.0	55	78	171	113	0.26
81–93	4.59	25.5	117	95	445	159	0.73
93–106	5.46	34.3	188	130	606	173	0.95
106–119	3.65	48.9	178	—	—	177	0.64
S122–123	2.00	43.3	87	91	126	149	0.30
S123–129	0	—	0	—	0	—	—
129–135	1.42	26.9	38	97	86	141	0.20
135–141	8.51	18.5	157	56	329	160	1.36
141–148	5.85	25.0	146	79	358	153	0.90
155–1.6 mg atropine							
148–160	3.82	21.9	85	112	357	197	0.75
160–171	3.46	55.7	193	—	—	191	0.66
171–181	3.00	40.2	121	155	423	200	0.60
S181–184	2.33	49.1	114	—	—	195	0.46
S184–186	4.00	55.5	222	153	432	206	0.82
186–188	1.50	60.2	90	168	190	199	0.30
188–193	1.45	46.2	67	139	146	215	0.31

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[1] 0 time represents beginning of control clearance collections. S represents submerged periods.

following the dive were considerably lower than before the dive and gradually returned toward normal.

The present data also show that the renal blood flow ceases during diving. If blood flow had continued, the renal capillaries would have continued to be perfused with plasma containing PAH. A maximum tubular gradient should be approached, as in a "stop-flow" study (Malvin, Wilde and Sullivan, '58), and one could expect the first urine obtained following the dive to have an increased concentration of PAH. This was not the case (table 1).

A decrease in glomerular filtration rate and renal blood flow during respiratory obstruction and asphyxia has been reported by Bradley and Bing ('42). Lowrence, Nickel, Smythe and Bradley ('56) found that nitrogen breathing had similar effects on renal hemodynamics. Their studies are not comparable to ours for three reasons. (1) Their animals were not trained to tolerate involuntary diving and consequently fought. As demonstrated earlier (Murdaugh, Seabury and Mitchell, '61) the cardiac response differs in the fighting seal from that of the quiet seal. (2) In both of their studies the asphyxia was interrupted during the so-called diving periods with the animal permitted to breathe every three to 5 minutes. In no case did they find complete cessation of urine production which seems reasonable since their collections represented mixed intervals of asphyxia and breathing. It is quite possible that urine flow ceased during the short periods of asphyxia because the creatinine U/P decreased in their studies as it did in our post-diving urine samples. (3) Irving, Scholander and Grinnell ('41) found that blood lactic acid concentration did not increase during 5 minute dives of trained seals, but rapidly increased during short periods of nitrogen breathing. This represents a striking difference in the seal suffering asphyxia and the seal undergoing an accustomed dive.

The decrease in renal concentrating ability following diving is of considerable interest. On basis of the countercurrent hypothesis it may be explained in the following manner. When glomerular filtration rate and renal blood flow are stopped
the countercurrent multiplier system ceas
to function. Consequently, the solute co
centration gradient in the kidney, esta
lished and maintained by the countercu
rent system, gradually decreases throu
diffusion. After the return of renal blo
flow and glomerular filtration rate it tak
a few minutes before the gradient is
stored and water reabsorption returns
predive values.

The natriuresis following diving m
possibly be caused by decreased sodiu
transport before the returning blood su
ply restores normal energy metabolism
the tubular cells.

Bradley and Bing ('42) reported th
atropine did not affect the renal respon
to respiratory obstruction. In the prese
study we found that atropine abolish
the renal response to diving in the train
seal. It is interesting that our results
the seal are similar to the findings of F
ster and Nyboer ('55) in the rabbit. Th
found that the decrease in renal blood fl
in response to voluntary apnoea[4] in t
rabbit is prevented by atropine.

Murdaugh, Mitchell and Wood (in pre
confirmed the finding that blood lac
acid does not increase during 5-minu
dives (Irving, Scholander and Grinne
'41), but found that following atropi
administration the blood lactic acid co
centration was increased at 4 minutes
diving. It is thus apparent that atropi
also impairs the arterial constrictor
sponse of diving. From the finding in t
seal that atropine prevents the cessati
of renal blood flow in response to divin
it becomes apparent that this renal
sponse is part of the overall arterial co
strictor response involving muscle, gu
and kidneys rather than a separate mec
anism.

SUMMARY

The clearances of exogenous creatini
and PAH were measured in young fema
harbor seals trained to allow submersion
the head for 10 minutes. In the traine
seal diving resulted in an immediate a
complete cessation of urine flow, glomer
lar filtration and by indirect evidence
cessation of renal blood flow. After t

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[4] Voluntary in the sense that the rabbit held
breath rather than smell strong odors.

e there was a decreased tubular reab-
ption of water and a stepwise return in
atinine and PAH clearances to normal.
ropine was found to block this renal
sponse to diving. The changes in renal
nction during diving by the seal may be
nsidered part of the arterial constrictor
sponse rather than an individual phe-
menon.

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Consideration of the Ribonucleic Acid epolymerase-Inhibitor Systems of ouse Tissues[1]

K. A. O. ELLEM[2] AND J. S. COLTER
The Wistar Institute of Anatomy and Biology, Philadelphia, Pa.

A considerable body of literature has
cumulated concerning enzymes which
ve nucleolytic activity. Their level and
stribution in the tissues of a number of
ammalian species have been measured
normal and abnormal conditions with
e object of correlating their activity with
own manifestations of cell function, and
elucidate their role in the cellular econ-
ny. In a preliminary note we have re-
rded the results of such an investigation
the tissues of the mouse (Ellem et al.,
9). Certain patterns in the ribonuclease
Nase) activity of the tissues were noted,
d from this a characteristic pattern of
Nase activity in a series of mouse ascites
mors was subsequently revealed (Colter
al., '61). The presence of naturally oc-
rring RNase inhibitors in tissue homog-
ates has been reported (Pirotte and
sreux, '52; Roth, '56; Colter et al., '61).
The previous investigation of the RNase
tivities of normal mouse tissues was
erefore extended in an attempt to evalu-
e the role of the several variables affect-
g the complex multi-enzyme-inhibitor
stems involved, and thus to shed some
ht on the problems inherent in studies
ribonucleolytic enzyme systems in tissue
mogenates. The results of these investi-
tions are presented here.

MATERIALS AND METHODS

RNase assay. The estimation of RNase
tivity was performed as previously de-
ribed (Ellem et al., '59; Colter et al., '61).
ie optimal ribonucleic acid (RNA) con-
ntration was determined for a number
tissues and found to be ⅓% for the
kaline RNase. At this substrate concen-
ation, the acid RNase activity of a num-
r of tissues was slightly inhibited. All estimations were carried out using a concentration of ⅓% RNA, and the values reported in table 1 for the alkaline activity:acid activity ratios are, except where indicated, corrected for this inhibition.

The values listed in table 1 were read from pH-activity curves, constructed from estimations of enzyme activity at 15–20 pH intervals between 5.0 and 9.0. To compare the results of several experiments in which the effects of various compounds on RNase activity were examined, mean curves were constructed. This was done by normalizing—graphically—the set of curves from each experiment. The maximum observed activity (usually a point from the curve obtained in the presence of p-chloromercuribenzoate, p-CMB) was given the arbitrary value of 100, and the activity at every other point expressed as a fraction thereof. The curves were then drawn, and RNase activities were read off at intervals of 0.1–0.15 pH units. These values (obtained from a number of experiments with each tissue) were averaged, and the means used to construct the mean curves, examples of which appear in figures 1, 3, 4 and 5. The data describing the effects of ethylene-diaminetetraacetic acid (EDTA) and p-CMB which are collected in tables 2 and 3 were read from such mean curves.

RESULTS

The data accumulated from an extension of the observations previously re-

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[2] Traveling fellow of the New South Wales Cancer Council; Wistar Institute trainee; Present address, Department of Bacteriology, University of Sydney, N.S.W., Australia.

ported (Ellem et al., '59) on the levels and pH optima of RNA depolymerizing activity in homogenates of mouse tissues are collected in table 1. Most tissues exhibited at least two maxima of activity. The previously suggested grouping of the tissues on the basis of the appearance of their pH-activity curves and the level of the alkaline enzyme, was preserved. In general, it is clear that the activities of the acid and alkaline ribonucleases, in tissues which contain both enzymes, are of the same order of magnitude. This is readily apparent from the last column, in which the values of the ratio of alkaline activity: acid activity are seen to fall in the range 0.5 to 1.7, despite the fact that the tissues may differ in total RNase activity by a factor as large as 100.

The pH-activity curves of thymus, lymph nodes, spleen and lung did not present clear-cut pyrimidal maxima. Their appearance suggested that perhaps three or more curves, having maxima between pH's 5 and 8, were superimposed, or that the activity of the enzymes in these tissues were less sensitive to alterations in pH over this range of H-ion concentration, and therefore gave rise to broad, truncated maxima (fig. 1).

In the case of erythrocytes and skelet muscle, RNase activity on the alkaline si of neutrality was very low with an i defined maximum in the region of pH 7 In homogenates of both tissues, the abili to degrade RNA to acid-soluble produc increased below pH 5.5, but in neither d a maximum occur at a pH above 4.5. T nature of this activity was pursued no fu ther, although it is not without interes

Removal of RNA depolymerase inhibiti activity. Roth ('56) has demonstrated t presence of an RNase inhibitor in rat liv homogenates, and, in this laboratory, t presence of such inhibitors has been dem onstrated in a number of ascites tumors mice (Ellem et al., '59; Colter et al., '61 Since these inhibitors can alter the sha of the pH-RNase activity curve of the ti sue in which they occur (Colter et al., '61 it was decided to examine the normal t sues of the mouse for their presence, usi the –SH reactive compound, p-chlorome curibenzoate (p-CMB) to destroy their a tivity (Roth, '56).

The addition of p-CMB to liver homo enates caused a considerable increase the activity of the alkaline (but less of t acid) nucleic acid depolymerizing activit Figure 2 illustrates the increase in liv

TABLE 1

RNA depolymerase activity of mouse tissue homogenates

Tissue	pH	Activity[1]	pH	Activity[1]	Alkaline activit / Acid activity
I. Pancreas (9)[2]	—	—	7.3	10,100 ± 2400	—
II. Spleen (8)	5.8	118 ± 35	7.0–7.4	146 ± 32	1.2
Intestinal mucosa (5)	5.6	47.6 ± 16.9	7.3–7.4	100 ± 13	1.7
Lymph nodes (5)	6.0	50.6 ± 7.5	7.3	70 ± 17	1.4[3]
Thymus (5)	5.8	40.9 ± 9.6	7.4–7.5	38.8 ± 9.3	0.95[3]
Lung (5)	5.6	16.5 ± 3.6	7.6–8.0	27.7 ± 6.5	1.7[3]
III. Kidney (7)	5.7	25.7 ± 4.1	8.1	36.1 ± 2.6	0.87
Salivary gland (3)	5.6	17.8 ± 1.5	7.5–7.9	19.8 ± 3.4	1.0
Liver (8)	5.7	21.8 ± 4.8	7.7	12.5 ± 4.6	0.5
IV. Brain (3)	5.9	6.0 ± 0.9	8.2–8.3	3.2 ± 0.1	0.5
V. Muscle (3)	< 5.0	> 1.2	7.5	1.6 ± 1.1	1.3
Erythrocytes (2)	< 4.5	> 4.0	7.5	0.36 ± 0.02	< 0.1
VI. Serum (2)	—	—	7.3	4.0 ± 0.3	—
Ascitic plasma (9)	—	—	7.1–7.3	3.5 ± 1.0	—

[1] Expressed as the increase in optical density at 260 mμ of the acid-soluble supernatant produc by 1 gm (wet wt.) of tissue in 30 minutes.

[2] Numbers in brackets indicate number of estimations performed.

[3] Not corrected for slight inhibition of acid RNase activity produced by substrate concentrati used in assay.

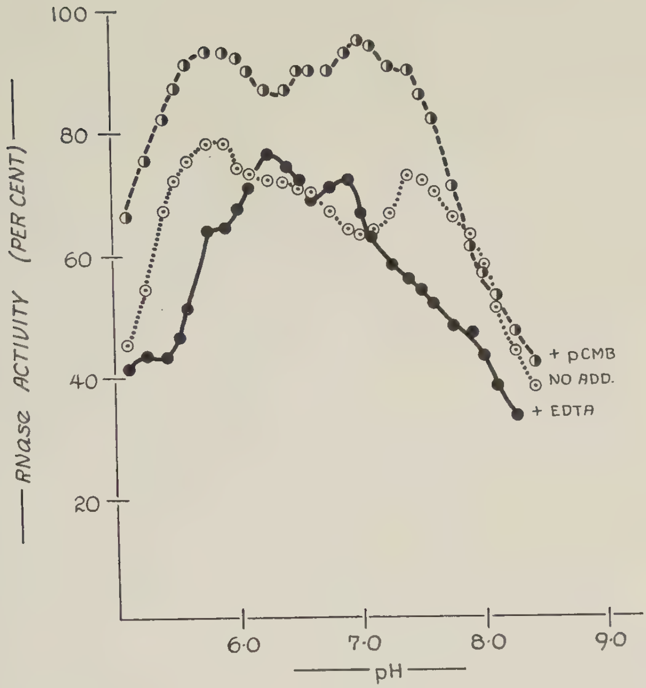
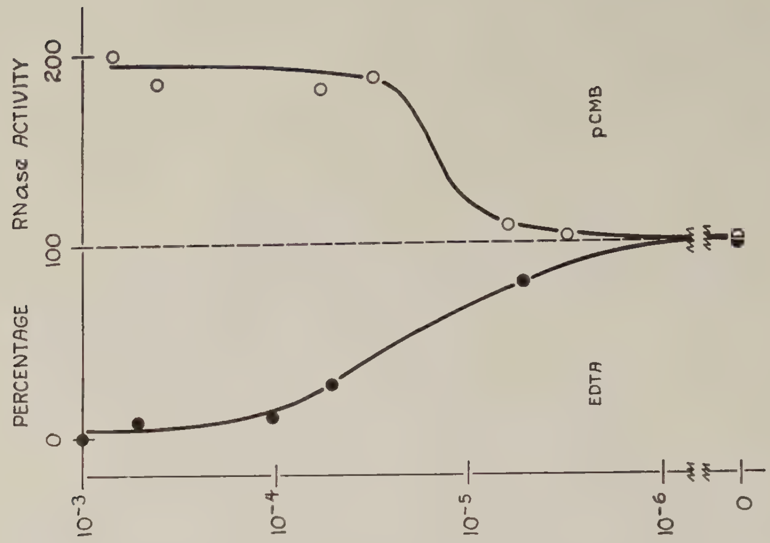


Fig. 1 pH-RNase activity curves of mouse thymus homogenate measured: ⊙, normal assay system (no additions); ◑, in the presence of 4 × 10^{-4} M p-CMB; ●, in the presence of 10^{-4} M EDTA. Ribonuclease activity is expressed as the percentage of the activity measured at the optimum pH with inhibitor immobilized by p-CMB.

kaline RNase activity with increasing
ncentrations of p-CMB, a concentration
tween 1 and 2 × 10^{-5} M being in the
itical region of response. It was there-
re decided to use p-CMB at a concentra-
n of 4 × 10^{-4} M in examining the effect
this compound on the RNase activity
other tissues. The results of these in-
stigations are summarized in table 2.
general, the higher the level of activity
RNase in a tissue, the smaller the in-
ease produced by the addition of p-CMB
the assay mixture. Also, more activa-
n was observed at pH 7.0 than at either
the pH maxima. Figures 1 and 3 dem-
nstrate that p-CMB, in addition to stimu-
ting enzyme activity, produces a change
the shape of the pH-activity curve. The
ost consistent change was a broadening
the alkaline peak of activity, due to a
arked increase in enzyme activity at pH
0. The curves obtained with homoge-
ates of thymus and liver are presented
re as being typical of the two major
oups of tissues (II and III in table 1).

Augmentation of inhibition. Since naturally occurring inhibitors in the tissues can profoundly alter the level and other characteristics of the RNase activity of a tissue homogenate, other factors in their expression were studied. If, for example, heavy metals with –SH binding capacity were present, they could affect the observed RNase activity. Although present in quantities well below those necessary to inhibit the enzyme, they could, by binding varying quantities of the inhibitor (which is very sensitive to these materials) (Roth, '56) release RNase from an inactive RNase-inhibitor complex and so stimulate the activity of the enzyme. The effects of the removal of traces of heavy metals were examined by adding the chelating agent, EDTA, to the tissue homogenates.

Initial experiments were performed with liver homogenates, and yielded the surprising observation that the presence of EDTA produced approximately 40% inhibition of activity in the acid region, and almost total suppression of activity at the alkaline



MOLARITY OF ADDED COMPOUNDS (EDTA, pCMB)

Fig. 2 The effects of p-CMB and EDTA on the alkaline ribonuclease activity of mouse liver homogenates. Ribonuclease activity is expressed as the percentage of the activity measured at the optimum pH in the absence of any additions to the normal assay system.

TABLE 2

Activation of ribonuclease activity of tissue homogenates by 4 × 10^{-4} M p-CMB

Tissue	Per cent increase at pH optima and at pH 7.0		
	Acid	7.0	Alkaline
Pancreas	—	0	0
Spleen	11	6	6
Lymph nodes	14	23	12
Brain	20	57	0
Thymus	19	63	23
Kidney	46	95	10
Lung	56	74	72
Liver	69	284	88
Muscle	—	104	55
Erythrocytes	—	600	—
Ehrlich ascites tumor cells	0	1500	16

TABLE 3

Inhibition of ribonuclease activity of tissue homogenates by 10^{-4} M EDTA

Tissue	Per cent inhibitio at pH optima	
	Acid	Alkali
Pancreas	—	0
Spleen	0	0
Lung	0	3
Lymph nodes	16	4
Ascitic plasma	—	14
Thymus	18	23
Kidney	35	100
Liver	41	100

pH optimum. From figure 2 it may be seen that the extent of inhibition of RNase activity in liver homogenates is dependent on the concentration of EDTA employed. Almost complete suppression of the alkaline activity occurred at 10^{-4} M EDTA, and for this reason this concentration was used in similar experiments with other tissues. The data obtained are collected in table 3.

While little effect was noted in those tissues with a high level of RNase activity, significant inhibition was observed in tho
with less activity (with the exception
lung tissue, which had a multi-compone
type of pH-activity curve). Complete i
hibition of the RNase activity of liver a
kidney occurred at that pH at which, in t
absence of EDTA, alkaline activity w
maximal. These observations are ill
trated graphically in figure 3.

To check that the inhibition produced
EDTA was due primarily to augmentati
of the inhibitor rather than to the bindi
of cations essential for enzyme functio
4 × 10^{-4} M p-CMB was added to liv

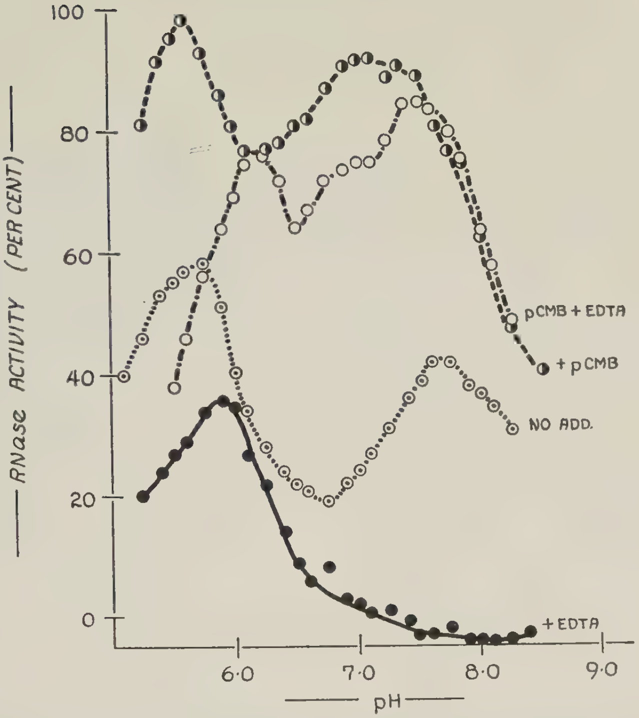


Fig. 3 pH-RNase activity curves of mouse liver homogenate measured: ⊙, normal assay system (no additons); ◑, in the presence of 4 × 10^{-4} M p-CMB; ●, in the presence of 10^{-4} M EDTA; ○, in the presence of 4 × 10^{-4} M p-CMB and 10^{-4} M EDTA.

omogenates already containing 10^{-4} M DTA. From figure 3 it may be seen that is manipulation restored most of the lkaline enzyme activity (to 85% of the ctivity measured in the presence of p-MB alone). The same effect was oberved with kidney, although the level of estoration of activity (some 60% of the naximal value) was lower. That some ther effects were present is clear from the lifference in shape of the p-CMB and p-MB + EDTA curves shown in figures and 3.

Neither p-CMB or EDTA altered, in any letectable fashion, the measurable activty or the shape of the pH-activity curve btained with either crystalline bovine ancreatic RNase or homogenates of nouse pancreas.

The mean curve derived from a number f determinations of the enzyme in ascitic lasma obtained from mice bearing the hrlich ascites carcinoma, exhibited a naximum closer to pH 7.1 than to the 7.3 previously reported (Ellem et al., '59). p-CMB caused little alteration in the level of this activity, but did cause a slight change in the pH-activity curve which suggested the existence of two maxima, very close together, one on either side of pH 7.0. EDTA, on the other hand, while having little effect on the activity at pH 7.3, caused a 14% inhibition at pH 7.0, and produced a sharpening of the alkaline maximum. It eliminated the component on the acid side of pH 7.0, with little or no alteration in the alkaline, descending limb of the curve (fig. 4).

It was previously shown that the combination of crystalline pancreatic RNase and the RNase inhibitor isolated from the Ehrlich ascites tumor is pH-dependent. Dissociation of the enzyme-inhibitor complex occurred below pH 5.0 and above pH 8.5. It might thus be expected that the complex is most stable, and the inhibition of RNase most complete, in the middle of this range—that is at pH 6.5–7.0. If we

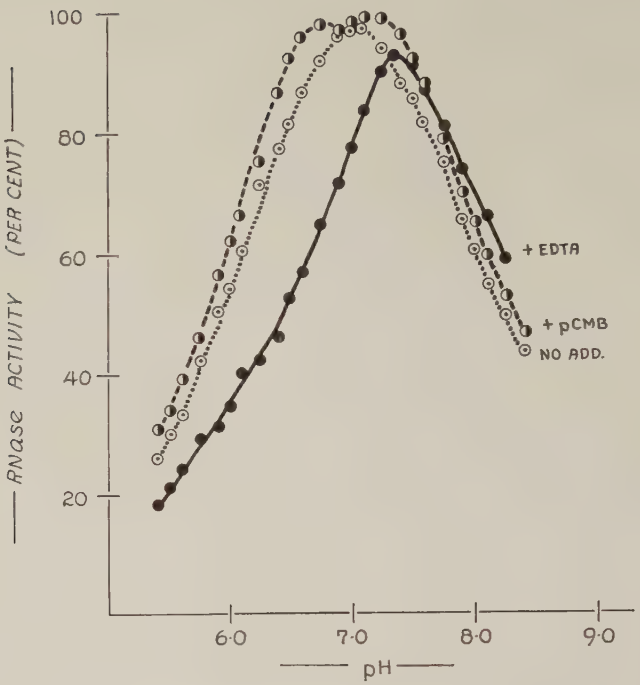


Fig. 4 pH-RNase activity curves of ascitic plasma from mice bearing the Ehrlich ascites tumor, measured: ⊙, normal assay system (no additions); ◑, in the presence of 4×10^{-4} M p-CMB; ●, in the presence of 10^{-4} M EDTA.

assume that p-CMB does not alter the pH characteristics of the tissue RNases—and it was, in fact, shown not to affect this characteristic of either bovine or mouse pancreatic RNase—then the difference curve, obtained by subtracting the standard pH-RNase activity curve from the curve obtained in the presence of p-CMB, should represent the dependence on pH of the combination between the tissue inhibitor and tissue RNases. Such difference curves, obtained from several tissues, are shown in figure 5.

Despite very marked differences in the shapes of the pH-activity curves of the various tissues, there was excellent agreement among the curves representing the difference between the uninhibited (in the presence of p-CMB) and partially inhibited (no additions) pH-activity curves. All had predominant maxima near pH 7.0, the height of which reflects the degree to which the enzyme is inhibited in the fresh homogenate. It is probable that this peak represents the dependence on pH of the enzyme-inhibitor interaction. If the inhibit combined with the enzyme independent of pH, the difference curves, as plotted figure 5, would be horizontal straig lines. The possibility that this peak at p 7.0 represents the activity of a third e zyme which is specifically, and exclusivel inhibited by the RNase inhibitor, seems be ruled out by the observation that EDT entirely abolishes the alkaline RNase a tivity of liver and kidney in a mann which is reversed by p-CMB.

DISCUSSION

The data presented here suggest a nu ber of conclusions regarding the distrib tion and activity of RNA depolymeras in the tissues of the mouse. Each tiss examined—with the sole exception of t pancreas—displayed at least two maxi when the enzyme activity was consider over a wide range of hydrogen ion conce tration. The picture obtained with pa creas was to be expected since the activi observed in homogenates of this tissue w

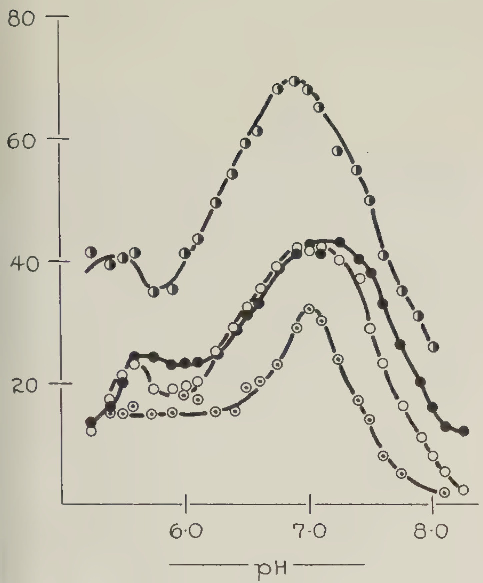


Fig. 5 Curves obtained by subtracting from e pH-RNase activity curves obtained in presce of p-CMB (RNase inhibitor immobilized) e pH-RNase activity curves obtained using the rmal assay system (RNase partially inhibited RNase inhibitor), with ◑, mouse liver hogenate; ●, mouse lung homogenate; ○, mouse lney homogenate; ⊙, mouse thymus homogate.

arly 100-fold greater than that shown homogenates of the next most active sue, when their activities were compared the basis of concentration of enzyme r unit of wet organ weight. This great tivity is caused by the elaboration of a ecific digestive enzyme (Rabinovitch, 4; Schucher and Hokin, '54) which is creted into the digestive juice. It might expected that, in addition to this enme, pancreas would possess the two her enzymes which have been found in e parenchymatous organs such as saliry gland, liver and kidney in the mouse able 1) and in rat liver and kidney (Roth, 4; de Lamirande et al., '54). The activity the "ordinary" acid and alkaline ribocleases would be completely masked in omogenates of pancreas by the high level the digestive enzyme. Dickman et al. 59, '60) have presented evidence for the esence of two and possibly three enmes in this organ.

It was previously suggested that the oupings of tissues on the basis of the level and pH activity characteristics of the alkaline RNase had some significance in terms of their physiological functions. It is of interest that the second group of tissues in table 1 have large amounts of lymphoid tissue associated with them, and all exhibit multi-component pH-activity curves. These tissues probably contain more than two enzymes having RNA depolymerase activity. Hilmoe and Heppel ('53) have distinguished three "ribonucleases" in splenic tissue. The exigencies of intestinal mucosa and lung have been discussed previously (Ellem et al., '59).

The relationship between the levels of activity of the acid and alkaline depolymerizing activities in the tissues of the mouse is, as a whole, one of approximate parallelism. Intestinal mucosa, lung, brain and liver deviated from this relationship, the two former having 1.7 times as much alkaline as acid activity and the latter two one-half as much. In the case of the intestine, an artefact introduced by the adsorption of RNase from the gut contents has been previously discussed (Ellem et al., '59). With liver, the level of alkaline RNase activity has been found to depend very much on the level of the RNase inhibitor present in this tissue. The brain alone, among all the tissues studied, appeared to have a very low level of alkaline RNase —a level not due to the presence of the p-CMB-sensitive inhibitor present in the other tissues (table 2). The correlation between the alkaline: acid RNase activities and the mitotic rate which has been suggested by deLamirande and Allard ('59) has not been confirmed in these investigations.

As is evident from figures 1, 3 and 4, the estimation of "ribonuclease" activity in tissue homogenates is affected by a number of variables. Probably the most important of these is the extent to which the naturally occurring inhibitor combines with the nuclease enzyme systems. Not only the level of activity, but the position of the pH optima of the enzymes is influenced by this inhibitor. Most reports in the literature—many of which were published prior to the detection of the inhibitor—do not evaluate the effects of this component. Quite clearly, however, in all attempts to evaluate the influence of variations in tissue physiology, and in efforts to establish correlations between nuclease activity and drug action, the presence of the inhibitor should be sought and quantitated. Perhaps a short cut to evaluating its influence on the system would be to perform RNase estimations at the known pH optima for the tissue under investigation and at pH 7.0 in the presence and absence of 4.10^{-4} M p-CMB and 10^{-4} M EDTA. If either of these agents significantly altered the levels of activity, then a fuller investigation of the system would be indicated. If not, then any observed alterations in the levels of activity at the pH optima might in fact indicate a true change in the level of the enzyme.

The evidence which we previously adduced for the pancreatic origin of serum ribonuclease has been considerably obscured by the present findings. Also, the effects of p-CMB and EDTA on the pH-activity curve of the ascitic plasma RNase (fig. 4) suggests that (1) either a significant amount of RNase inhibitor is circulating in this fluid, or, more probably (2) a second RNA-splitting enzyme is present, dependent upon divalent cations for its activity, with a pH optimum at about 6.5 (as determined from the difference between the p-CMB and EDTA curves in fig. 4).

Rabinovitch and Dohi ('56) have presented evidence which argues against a pancreatic origin for the serum RNase of the rat. By teleological speculation, Herriott et al. ('61) have attributed to serum nucleases a role in destroying endogenously produced infectious nucleic acids. They consider the serum enzyme to be a supplement to the immunological defenses of the host, which operate only against intact virus. The practical effects of extra-cellular RNase in preventing *in vivo* infection with exogenously administered infectious viral RNA preparations have been described elsewhere (Ellem et al., '59; Colter and Ellem, '60).

This report has considered in the same category, all the enzymes in a tissue which exhibit RNA depolymerizing activity. Although this includes a number of enzymes with different substrate specificities, it was done for several reasons. Althou attacking different linkages in the RN molecule, these enzymes are still all al to effect some degradation—and perha some exchange and synthetic reactio within this class of compound (Hepp and Whitfield, '55; Heppel et al., '55 They may all, therefore, be concerned RNA metabolism within the cell. T broad spectrum of this activity in the t sues from a single species was obtain in an attempt to correlate the findin with what has previously been describ only in isolated tissues from different sp cies of animals. Finally, a considerati of the variables involved in the RNa RNase inhibitor systems is most importa in attempting to evaluate investigatio which purport to discover variations in t tissue RNases following alterations in t milieu of the animal.

However, an increasing degree of phistication is developing in the investi tion of RNases. The acid and alkali enzymes of various tissues have be found to have different modes of acti (Hilmoe and Heppel, '53; de Lamiran and Allard, '59; Nodes and Reid, '58 cleaving phosphodiester bonds of differe specificity in the RNA molecule. This d ference has, in fact, been the basis fo method for the separate estimation of t acid and alkaline enzymes occurring gether (Nodes, '58). Spleen and intesti have been shown to be able to degra RNA by non-specific phosphodiesteras (Heppel and Hilmoe, '55). These can detected by their ability to cleave all esters of purine nucleotides to the 3′ mon nucleotides, or to digest the RNase-resi ant "core" polynucleotide. Clearly, these facets must be considered in inv tigations designed to examine the functi of the RNA depolymerase systems by c relating their variation with changes duced in the animal or tissue under co sideration.

The physiological function of the cell lar ribonucleases is still unknown. T acid RNase appears to exist in the ly somes, and is presumed to have a functi comparable to that of the other acid hyd lases present in these particulates (Duve, '59). Roth ('60) has made t

eresting observation that the activity of
e alkaline RNase present in rat liver
crosomes must be intact for the incor-
ration of L-leucine-C^{14} into the micro-
mal protein.

The RNase inhibitor affects the alka-
ie enzyme of homogenates primarily
g. 3 and Roth, '59). This is, however,
obably a result of the dependence on pH
the enzyme-inhibitor combination, and
hough it little affects the acid enzyme
its optimum pH (5.5), it may inhibit
at pH 7.0. If it has any function as a
Nase inhibitor in the cell—and is not
erely an artefact produced by tissue
mogenization—then its occurrence in
e cell supernatant (Roth, '58) together
th inhibitors of other hydrolases (de
ive, '59), suggests that it may play a
otective role against the degradative ef-
cts of these enzymes in the event of their
ing liberated by lysosomal breakdown.

SUMMARY

The tissues of the mouse have been
amined for the presence of ribonucleic
id depolymerases, and have been di-
ded into groups on the basis of their
H-RNase activity curves and the level of
tivity of their alkaline RNase. The pH
otima (two for most tissues) and the
nzyme activity at these optima are docu-
ented.

The effects of several variables which
fluence the RNase activity of tissue ho-
ogenates have been examined, with em-
nasis on the effect of p-CMB on the
nzyme activity. This compound, which
mbines specifically with a naturally oc-
urring RNase inhibitor, not only stimu-
ites to varying degrees the alkaline RNase
ctivity of mouse tissues, but modifies the
hapes of the pH-activity curves. It seems
ear that the measured RNase activity
f any tissue is strongly influenced by the
xtent to which the enzyme is combined
ith the naturally occurring inhibitor.

EDTA, at a concentration of 10^{-4} M
as been shown to abolish completely the
lkaline RNase activity of mouse liver and
idney homogenates, and to inhibit to a
esser degree the RNase activity of other
issues. The mechanism of this inhibition
ppears to be that the EDTA liberates
Nase inhibitor from inactive complexes
with traces of heavy metals—the freed inhibitor in turn combining with the depolymerase. This conclusion is supported by the observation that the addition of p-CMB to homogenates already inhibited by EDTA restores the activity to almost normal levels.

An analysis of the pH-RNase activity curves obtained in the presence and absence of p-CMB indicates that the inhibitor and the enzyme are most tightly bound at pH 7.0, with the complex showing an increasing tendency to dissociate as the pH is either raised or lowered from neutrality.

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